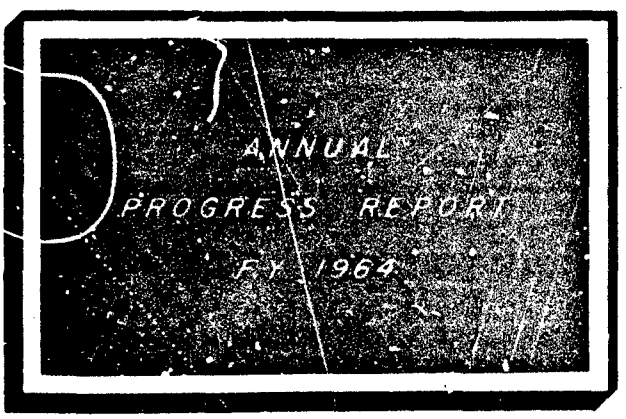
UNITED STATES ARMY MEDICAL UNIT

MEDICAL RESEARCH AND DEVELOPMENT COMMAND——WALTER REED ARMY MEDICAL CENT

FORT DETRICK, MARYLAND



RCS-MEDDH-288

UNITED STATES ARMY MEDICAL UNIT

ANNUAL PROGRESS REPORT

FISCAL YEAR 1964

RCS-MEDDH-288

FOREWORD

This FY 1964 Annual Progress Report is a general review of the research activities of the U. S. Army Medical Unit, Fort Detrick, Maryland, under Project 1C622401A096 (formerly 1C012501A030), Medical Defense Aspects of Biological Warfare (U) (formerly Risk to U. S. Armed Forces from Biological Warfare). The project is divided into three Tasks as follows:

1C622401A096-01 - Vulnerability of Man to Biological Warfare.
1C622401A096-02 - Prevention and Treatment of Biological Warfare Casualties.
1C622401A096-03 - Laboratory Identification of Biological Warfare Agents.

Tasks 01 and 02, have Subtasks represented by contractual agreements with industrial firms or educational institutions. Their reports are available through DDC.

Tasks are internally subdivided into Studies, identified by a four-digit suffix. The first two digits are assigned as follows:

General	-00- to -09-
Bacterial Diseases	-10- to -29-
Rickettsial Diseases	-30- to -39-
Viral Diseases	-40- to -79-
Intoxications	-80- to -89-
Mycotic Diseases	-90- to -99-

The remaining two digits are assigned sequentially as specific studies are made parts of the research program.

When a study is completed or terminated its number is not reassigned. In the Table of Contents, Studies in these categories are so designated. COMPLETED indicates no further work is planned. TERMINATED indicates no further work is planned until additional personnel or facilities are available.

Dan Crozier

DAN CROZIER
Colonel, MC
Commanding

1 July 1964

ABSTRACT

A report of progress on the research program of the U. S. Army Medical Unit, Fort Detrick, Maryland, for Fiscal Year 1964 is presented.

ANNUAL PROGRESS REPORT

TABLE OF CONTENTS

PROJECT NO. 1C622401A096: MEDICAL DEFENSE ASPECTS OF BIOLOGICAL WARFARE (U)

PAGE

FOREWORD iii

ABSTRACT iv

INDEX BY DISEASE x

TASK NO. 1C622401A096-01: VULNERABILITY OF MAN TO BIOLOGICAL WARFARE

-01-00- GENERAL

-01. Chemical Constitution and Structure of Bacterial Cell Walls. COMPLETED. Transferred to Hood College Contract No. DA-49-193-MD-2604, Subtask -01-02.

-02. The Effect of Infectious Disease on Protein Metabolism. 1

-03. Kinetics of Virus Cell Interaction. TERMINATED--1963.

-04. Consequence of Selected Viral Infections in Man. 5

-05. Problems of Cross-Infection in Medical Treatment Facilities. TERMINATED--1963.

-06. Metabolic Balance Studies in Experimental Human Infectious Disease. 9

-07. Production, Transport, and Metabolic Degradation of Adrenocortical Hormones in Infectious Disease. 13

-08. Biochemical and Physiological Response to Biological Active Macromolecules. COMPLETED. Reported in Technical Memorandum 56, "Characteristics and Biological Effects of Staphylococcal Enterotoxin B (U)," Consolidated Report of Various Divisions and U. S. Army Medical Unit, Fort Detrick, Maryland. To be published. SECRET.

-09. Influence of Irradiation on Infectious Diseases. 17

-01-10- TULAREMIA

-01. Quantitation of Human Susceptibility to Infection with Aged Aerosols of Pasteurella tularensis. 39

-02. Comparative Pathology of Respiratory Infection with Pasteurella tularensis. TERMINATED--1963.

-01-20- ANTHRAX

-01. The Mechanism of Death in Anthrax in the Guinea Pig. 43

-02. The Effect of Egg Yolk and Various Phosphatides upon the Susceptibility of the Rat to Anthrax. 49

-03. The Effect of Protein Deficiency upon the Susceptibility of the Rat to Anthrax. 53

-04. The Effect of Anthrax on Cellular Metabolism. 57

-05. Biophysical Studies of Bacillus anthracis and Its Metabolites. 61

-06. Modification of Anthrax by Ionizing Radiation. TERMINATED--1963.

-01-40- VENEZUELAN EQUINE ENCEPHALOMYELITIS

-01. The Effect of Virus Infection on Host Cell Energy Metabolism. 67

-02. The Effect of Virus Infection on Host Cell Protein Metabolism. 71

-03. Biophysical Studies of Venezuelan Equine Encephalomyelitis. 75

-04. Modification of Response of Macaca mulatta to Venezuelan Equine Encephalomyelitis by Antibody Administration. 83

-05. Role of Antibody in the Clinical Manifestations of Venezuelan Equine Encephalomyelitis Infection. 87

-06. Effect of Total Body Ionizing Irradiation on the Response of Macaca mulatta to Venezuelan Equine Encephalomyelitis Infection. TERMINATED--1962.

-07. Neurophysiological and Behavioral Manifestations of Venezuelan Equine Encephalomyelitis Infection. TERMINATED--1962.

-08. Host-Parasite Relationships in Venezuelan Equine Encephalomyelitis. 99

-01-80- STAPHYLOCOCCAL ENTEROINTOXICATION

Technical Memorandum 56, "Characteristics and Biological Effects of Staphylococcal Enterotoxin B (U)," Consolidated Report of Various Divisions and U. S. Army Medical Unit, Fort Detrick, Maryland. To be published. SECRET.

TASK NO. 1C622401A096-02: PREVENTION AND TREATMENT OF BIOLOGICAL WARFARE CASUALTIES

-02-00- GENERAL

-01. Chemotherapeutic Modification of the Immune Response. 123

-02. Evaluation of Efficacy of Experimental Vaccines. 127

-03. Chemoprophylaxis and Therapy of Infectious Diseases of Potential Biological Warfare Significance. 141

-04. Studies in Combined Antigens. 165

-02-10- TULAREMIA

-01. Efficacy of Aerogenic Immunization of Man with LVS Tularemia Vaccine. COMPLETED. Transferred to University of Maryland Contract No. DA-49-007-MD-751, Subtask -01-01.

-02-11- TYPHOID FEVER

-01. Pathogenesis and Immunity in Typhoid Fever. COMPLETED--1962.

-02-12- PLAGUE

-01. Development and Evaluation of an Effective Vaccine Against Pneumonic Plague. 177

-02-20- ANTHRAX

-01. Efficacy of Vaccination in Anthrax Infections. 183

-02. Interaction of Prior BCG Experience and Respiratory Anthrax. COMPLETED--1963.

-02-40- VENEZUELAN EQUINE ENCEPHALOMYELITIS

-01. Reduction of Reactogenicity of Attenuated Vaccine Virus. 189

-02. Aerogenic Immunization with Attenuated Venezuelan Equine Encephalomyelitis Virus. 195

-03. Cross Immunity within the "A" Group of Arboviruses. COMPLETED. Transferred to Johns Hopkins University Contract No. DA-49-193-MD-2398, Subtask -02-02.

-04. Modification of Berge Vaccine Virus by Mammalian, Avian, and Arthropod Transmission. COMPLETED--1962.

-05. Influence of Total Body Ionizing Irradiation on Response to Living Viral Vaccines. 201

-02-50- GROUP B ARBOVIRUSES

-01. Studies with Selected Members of Group B Arboviruses. 205

-02-80- STAPHYLOCOCCAL ENTEROINTOXICATION

Technical Memorandum 56, "Characteristics and Biological Effects of Staphylococcal Enterotoxin B (U)," Consolidated Report of Various Divisions and U. S. Army Medical Unit, Fort Detrick, Maryland. To be published. SECRET.

TASK NO. 1C622401A096-03: LABORATORY IDENTIFICATION OF BIOLOGICAL WARFARE AGENTS

-03-00- GENERAL

-01. Identification of Airborne Particulates by Fluorescent Antibody Techniques. 215

-02. Identification of Microbial Pathogens by Fluorescent Antibody Techniques. 219

-03. Identification of Viral Agents in Short Time Incubation Tissue Culture. Consolidated with -03-00-02.

-04. Detection of Circulating Antigens by Chromatographic Methods. 225

-05. Diagnosis of Viral Infections with Homologous Bone Marrow Cultures. 229

-06. Study of Selected Arthropod Vector-Host Relationships by Immunologic Methods. 237

-07. Preparation and Use of Specific Fluorescent Antibodies. 241

-03-10- TULAREMIA

-01. Liquid Mediums for Isolation and Propagation of Pasteurella tularensis. COMPLETED--1962.

-02. Solid Medium for Isolation and Propagation of Pasteurella tularensis. COMPLETED--1962.

-03. Definition of Nutritional Requirements, Specific Metabolic Activities and Determinants of Virulence of Pasteurella tularensis. 245

-04. Metabolic Requirements of the Vaccine Strain (LVS) of Pasteurella tularensis. Consolidated with -03-10-03.

-05. Serologically Demonstrable Response to Antigens of Pasteurella tularensis. 249

-03-20- ANTHRAX

-01. Characterization of Strains of Bacillus anthracis that Differ in Biologic Activity. 253

-02. In Vitro-Produced Anthrax Toxin. 263

-03-30- RICKETTSIOSES

-01. Tissue Culture Studies with Rickettsiae. 275

-03-40- VENEZUELAN EQUINE ENCEPHALOMYELITIS

-01. Development of Inactivated Serologic Reagents. 279

-02. Development of Serologic Tests for Early Identification of Viral Diseases. 285

-03. Determination of Distribution of Viral Agents in Host Tissues Employing Radioactive Labeling Techniques. 295

-04. Separation, Purification, and Concentration of Arbovirus Agents and Antigen-Antibody Complexes. 299

APPENDICES

A. U. S. Army Medical Unit Guest Lecture Series. 303

B. U. S. Army Medical Unit Professional Staff Meetings. 305

Distribution List 307

INDEX BY DISEASE

ANTHRAX	43-66, 183-188, 253-276
GROUP B ARBOVIRUSES	205-210
PLAGUE	177-182
RICKETTSIOSES	275-278
TULAREMIA	39-42, 245-252
VENEZUELAN EQUINE ENCEPHALOMYELITIS	67-122, 189-204, 279-302

ANNUAL PROGRESS REPORT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-01:	Vulnerability of Man to Biological Warfare
Study No. -00-02:	The Effect of Infectious Disease on Protein Metabolism
Reporting Installation:	U.S. Army Medical Unit Fort Detrick, Maryland
Division:	Physical Sciences Division
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Authors:	Irving Gray, Colonel, MSC Gerald J. Crawley, 1st Lieutenant, VC
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-01: Vulnerability of Man to Biological Warfare

Study No. -00-02: The Effect of Infectious Disease on Protein Metabolism

Reporting Installation: U.S. Army Medical Unit
Fort Detrick, Maryland

Division: Physical Sciences Division

Period Covered by Report: 1 July 1963 to 30 June 1964

Professional Authors: Irving Gray, Colonel, MSC
Gerald J. Crawley, 1st Lieutenant, VC

Reports Control Symbol: RCS-MEDDH-288

Security Classification: UNCLASSIFIED

Protein metabolism was studied in chimpanzees using L-methionine-S^{35} incorporation into plasma albumin and globulin as a measure of protein turnover. In each of 3 chimpanzees, protein turnover was measured at normal body temperature, then during 2 periods in which rectal temperature was raised to 106F by externally applied heat, and the first during hyperthermia alone and the last during infection with Venezuelan equine encephalomyelitis virus. Protein breakdown was increased during hyperthermia with or without infection. Increased protein breakdown present with fever and with infection appear to contribute to the observed increase in nitrogen excretion in these conditions. Certain findings suggested the concept that protein synthesis as well as breakdown were stimulated by the presence of virus infection.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-01: Vulnerability of Man to Biological Warfare

Study No. -00-02: The Effect of Infectious Disease on Protein Metabolism

Description:

To determine the effects of hyperthermia on protein turnover in the chimpanzee. With the aid of S-35 labeled methionine, protein turnover rates are determined at normal temperature, at hyperthermia alone (106F), and with a virus agent in the presence of equal hyperthermia.

Progress:

The existing work has been prepared for publication; the paper has been accepted for presentation at the June 1964 Army Science Conference. Physically induced hyperthermia caused a marked decrease in the uptake of labeled methionine into plasma globulin in 2 of 3 chimpanzees. There were no observable changes in the uptake of methionine into albumin observed under the conditions of the experiment. When the same degree of hyperthermia was maintained in the presence of virus, there was an obvious increase in protein turnover. In all 3 experimental situations - control, hyperthermia, hyperthermia plus infection, the significant uptake of the Me-S^{35} occurred in the first 4 hours following administration of the tracer. Since the breakdown rate of tagged globulin during fever alone was the same as with fever during viral infection, then the increased uptake of the Me-S^{35} in the presence of virus appeared due to increased synthesis or uptake during the infection.

Summary and Conclusions:

Protein metabolism measured by L-methionine-S^{35} incorporation into plasma albumin and globulin in chimpanzees has been followed in conditions of normal body temperature, hyperthermia and hyperthermia plus virus.

Breakdown is increased in both experimental groups over that in the control group. It is concluded that this increase, caused primarily by the fever, contributed to the previously observed increased nitrogen excretions.

Evidence supports the concept that protein synthesis is stimulated by the presence of virus at a time simultaneous with that of the observed protein breakdown.

Publications:

None.

Presentations:

1. Crawley, G., Gray, I., and Hildebrandt, P.: "The Effect of Hyperthermia on Protein Turnover in Infection." Accepted for presentation at the Army Science Conference, June 1964.

ANNUAL PROGRESS REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biolgocial Warfare (U)

Task No. 1C622401A096-01: Vulnerability of Man to Biological Warfare

Study No. -00-04: Consequence of Selected Viral Infections in Man

Reporting Installation: U.S. Army Medical Unit
Fort Detrick, Maryland

Division: Medical

Period Covered by Report: 1 July 1963 to 30 June 1964

Professional Author: William D. Sawyer, Major, MC

Reports Control Symbol: RCS-MEDDH-288

Security Classification: UNCLASSIFIED

ABSTRACT

Project No. 1C622401A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1C622401A096-01: Vulnerability of Man to Biological Warfare

Study No. -01-04: Consequence of Selected Viral Infections in Man

Reporting Installation: U.S. Army Medical Unit
Fort Detrick, Maryland

Division: Medical

Period Covered by Report: 1 July 1963 to 30 June 1964

Professional Author: William D. Sawyer, Major, MC

Reports Control Symbol: RCS-MEDDH-288

Security Classification: UNCLASSIFIED

No work under this task was accomplished during the reporting period.

BODY OF REPORT

Proejct No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-01: Vulnerability of Man to Biological Warfare

Study No. -00-04: Consequence of Selected Viral Infections in Man

Description:

To study the effects of selected virus diseases in man from a combined clinical and laboratory approach.

Progress:

No work under this task was accomplished during the reporting period.

Summary:

No work under this task was accomplished during the reporting period.

Publications:

None

ANNUAL PROGRESS REPORT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-01:	Vulnerability of Man to Biological Warfare
Study No. -00-06:	Metabolic Balance Studies in Experimental Human Infectious Disease
Reporting Installation:	U.S. Army Medical Unit Fort Detrick, Maryland
Division:	Physical Sciences Division
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Author:	William R. Beisel, Lt Colonel, MC
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-01: Vulnerability of Man to Biological Warfare

Study No. -00-06: Metabolic Balance Studies in Experimental Human Infectious Disease

Reporting Installation: U.S. Army Medical Unit
Fort Detrick, Maryland

Division: Physical Sciences Division

Period Covered by Report: 1 July 1963 to 30 June 1964

Professional Author: William R. Beisel, Lt Colonel, MC

Reports Control Symbol: RCS-MEDDH-288

Security Classification: UNCLASSIFIED

Metabolic balance studies have been utilized to study the impact of experimentally induced acute infection on the metabolism of human volunteers. A constant liquid plus fruit diet, adequate in all basic components, was employed with appropriate equilibration and control collections prior to challenge. Chemical analyses have been completed in groups of men exposed to an aerosol of Pasteurella tularensis, or to artificial hyperthermia induced by a hot humid environment and in another group by bacterial endotoxin given intravenously. Analyses are in progress on subjects exposed to Coxiella burnetii and in controls who received antibiotic therapy only. During acute tularemia there was a prolonged cumulative loss of N, K, P, and Mg. The cumulative losses of these intracellular elements were not repleted far into convalescence despite the prompt use of specific antibacterial therapy. The severity of the disease was related to the magnitude of the loss. Artificial hyperthermia, induced by a hot humid environment, produced certain changes which were different from those seen in acute infection, but many of the observed changes suggested that fever, per se, was indeed a contributing factor to the metabolic alterations associated with acute infection.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-01: Vulnerability of Man to Biological Warfare

Study No. -00-06: Metabolic Balance Studies in Experimental Human Infectious Disease

Description:

To study changes in nitrogen, electrolyte and mineral balance in man induced by acute experimentally induced infection (or in appropriate control situations), and to correlate observed metabolic changes with various clinical and laboratory features of the infection.

Progress:

The study of metabolic responses of the human host to acute infection has been pursued with considerable activity. Methods of diet preparation, specimen collection, and analysis have become well standardized. The laboratory personnel required to conduct these studies have been developed with on-the-job training. In addition to the 16 subjects exposed to Pasteurella tularensis who were studied initially, other groups have been studied using the metabolic balance technique. These include 8 subjects exposed to artificial hyperthermia to produce fever similar to that seen in tularemia, 6 subjects who received intravenous bacterial endotoxin to produce fever, 8 subjects exposed to Coxiella burnetii, and 8 non-exposed subjects who were treated with antibiotics (streptomycin or tetracycline) to obtain control data concerning the metabolic effects of therapy. Another study involving 8 men is in progress to determine if the source of dietary protein (animal vs. vegetable) exerts an influence on the metabolic response to acute tularemia.

Laboratory analyses have been completed in 3 of the above projects and are in various stages of completion in the others. Balance data for Na, K, Cl, Ca, PO_4, Mg, and N and nitrogenous components which include urea, creatinine, uric acid and alpha amino acid nitrogen have been calculated in 16 subjects exposed to acute tularemia.

With the onset of symptoms and fever in subjects with acute tularemia there developed a loss of N, K, P, and Mg which appeared to be due in part both to increased urinary excretion and to decreased dietary intake. Cumulative loss of these intracellular elements persisted throughout the course of the acute illness and recovery. There was no repletion of these losses far into convalescence despite the prompt use of specific streptomycin therapy. The severity of the disease appeared related to the magnitude of loss. Extracellular electrolytes, Na and Cl, were lost in excess during the onset of acute

illness, but losses were promptly reversed by renal retention; changes in Ca were only minimal. Urinary N breakdown products showed increases with illness which varied sufficiently in the timing to give indications that changes were taking place in the utilization of various pathways of intermediate nitrogen metabolism.

Artificially induced hyperthermia was seen to show both similarities and differences in the handling of minerals and electrolytes from those observed during acute infection. These studies were conducted in conjunction with Major Robert J.T. Joy and Dr. Ralph Goldman of the USA Research Institute of Environmental Medicine, Natick, Massachusetts. Because of large losses of sweat, replaced on a volume basis by water alone, dilutional changes were noted in the plasma electrolytes. Large sweat losses of Na, Cl, K, Ca, Mg and urea contributed to negative blaances of these elements. The hot atmosphere induced hyperventilation with resultant respiratory alkylosis of modest degree. This in turn lead to an exaggerated fall in serum P and the virtual disappearance of P from urine and sweat, and so prevented occurrence of a negative P balance. Analytic procedures of studies involving endotoxin fever, antibiotic therapy only, and Q fever are in various stages of completion.

Summary and Conclusions:

Metabolic balance studies have been pursued actively in groups of volunteers exposed to infectious agents or to various controlled conditions. Extensive chemical analyses have been completed or are in progress in complete metabolic balance studies involving 52 individuals.

These studies are providing basic information on many metabolic changes which accompany experimentally induced infection in humans. These same parameters are being correlated with studies of adrenal function and with various other clinical, bacteriologic and laboratory data.

Publications:

1. Beisel, William R., and Sawyer, W.C.: "Metabolic Balance Studies during Experimental Tularemia in Humans," Clin Res 12: 36, 1964 (abstract).

2. Beisel, W.R., Sawyer, W.D., Bruton, J., Gray, I., and Crozier, D.: "The Effect of Experimentally Induced Acute Tularemia in Humans on Nitrogen, Mineral, and Electrolyte Metabolism and on Adrenocortical Function." Program of the 45th Annual Session, American College of Physicians, p. 70, 1964 (abstract). Presented 10 April 1964.

ANNUAL PROGRESS REPORT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-01:	Vulnerability of Man to Biological Warfare
Study No. -00-07:	Production, Transport, and Metabolic Degradation of Adrenocortical Hormones in Infectious Disease
Reporting Installation:	U.S. Army Medical Unit Fort Detrick, Maryland
Division:	Physical Sciences Division
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Author:	William R. Beisel, Lt Colonel, MC
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-01: Vulnerability of Man to Biological Warfare

Study No. -00-07: Production, Transport, and Metabolic Degradation of Adrenocortical Hormones in Infectious Disease

Reporting Installation: U.S. Army Medical Unit
Fort Detrick, Maryland

Division: Physical Sciences Division

Period Covered by Report: 1 July 1963 to 30 June 1964

Professional Author: William R. Beisel, Lt Colonel, MC

Reports Control Symbol: RCS-MEDDH-288

Security Classification: UNCLASSIFIED

Studies to delineate and quantitate various aspects of the adrenocortical response to the stress of experimentally induced infection or to febrile states were continued. A total of 72 volunteers have been followed. These studies to date have shown an increasing excretion of adrenocortical hormones with the onset of developing tularemia and an abrupt return to normal after institution of therapy. Similar changes were observed in persons subjected to artificial hyperthermia induced physically by a hot, humid environment. It was observed in the subjects with tularemia that the degree of severity of the clinical illness was proportional to the increase in adrenocortical output. Changes were most marked in the 17-hydroxycorticoid fraction but were also present within the pregnanetriol and 17-ketosteroid metabolites. Samples from volunteers subjected to Q fever or antibiotic therapy alone are undergoing analysis.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-01: Vulnerability of Man to Biological Warfare

Study No. -00-07: Production, Transport, and Metabolic Degradation of Adrenocortical Hormones in Infectious Disease

Description:

To obtain information on production rates, plasma transport mechanisms, and probable qualitative and quantitative changes in the urinary excretion of adrenocortical hormones and their metabolites in association with acute infectious disease, with especial emphasis on the incubation and prodromal periods.

Progress:

Collections of blood and urinary steroids have been obtained during 42 studies in human subjects exposed to tularemia which included non-immunized, sham-exposed, and immunized, and previously infected volunteers. Quantitation of the adrenal steroid metabolites in urine and of plasma cortisol are being conducted in the steroid laboratories of the Department of Metabolism, Walter Reed Army Institute of Research. Plasma binding is being determined in this laboratory. An increase in the output of 17-hydroxycorticoids appeared to parallel the increasing symptoms and fever of acute tularemia, but abruptly returned to normal following institution of streptomycin therapy. This increase was proportional to the extent and severity of clinical infection and was only minimal, short-lived, or entirely absent in the immunized or previously infected volunteers who showed slight symptoms only. No changes in output were noted in the 2 sham-exposed individuals.

Measurements of urinary pregnanetriols showed a tendency toward increased excretion during the earliest days of symptoms and fever in acute tularemia. The urinary 17-ketosteroid metabolites showed a major increase during the height of illness which then dropped abruptly to normal with institution of therapy. Fractionation of the 17-ketosteroid metabolites generally showed that a reversal of pattern took place. Normally androsterone, etiocholanolone, and dihydroepiandrosterone fractions are excreted quantitatively in that order. With fever due to acute tularemia the dihydroepiandrosterone fraction increased markedly and became quantitatively greater than the other fractions in most subjects.

The studies of plasma cortisol and its binding during infection are in progress. In relating the timing of adrenocortical change to those of observed alterations in metabolic balance, especially those of

nitrogen and electrolytes, it appeared that the increase in glucocorticoid excretion preceded major changes in nitrogen balance. It also appeared that glucocorticoid excretion had normalized several days before a renal conservation of Na and Cl became maximal. Because of this latter observation, additional studies are in progress to determine the exact time sequence of glucocorticoid versus mineralocorticoid output.

Because of the abrupt return of excess glucocorticoid excretion to normal with the institution of therapy, a group of 8 men have been studied during antibiotic treatment without exposure to diseased organisms. Results on these studies are pending. Another group of 8 men with fever induced by a hot humid environment (studied in conjunction with Major Robert J.T. Joy and Dr. Ralph Goldman, USA Research Institute of Environmental Medicine, Natick, Mass.) showed changes in hydroxysteroid excretion which were similar in magnitude to those seen in the acutely ill tularemia patients. In this group a reversal of the usual diurnal pattern of steroid excretion was observed. Six men receiving bacterial endotoxin intravenously developed only short-lived mild fever and failed to show an adrenal response as evidenced by corticoid metabolite excretion measurements. Adrenocortical studies on 8 subjects who developed Q fever are pending.

Summary and Conclusions:

Results to date in large numbers of human subjects exposed to infectious organisms, or studied during other induced fevers, have shown that an increase in adrenocorticoid output appears to begin with the onset of fever and to disappear with the institution of therapy. Changes in the fractional pattern of ketosteroid metabolite excretion have been documented. Studies of other infectious processes and control situations are in progress. Patterns of adrenal response can be compared temporally with various changes in balance on nitrogen, minerals, and electrolytes. These suggest that the considerable differentiation in the timing of glucocorticoid response from that of the mineralocorticoid response may be possible.

Publications:

1. Beisel, William R., Bruton, J., and Sawyer, W.D.: "Adrenocortical Responses During Acute Tularemia Induced Experimentally in Humans," Clin Res 11: 401, 1963.

2. Beisel, W.R., Sawyer, W.D., Bruton, J., Gray, I., and Crozier, D.: "The Effect of Experimentally Induced Acute Tularemia in Humans on Nitrogen, Mineral, and Electrolyte Metabolism and on Adrenocortical Function." Program of the 45th Annual Session, American College of Physicians, p. 70, 1964 (abstract). Presented 10 April 1964.

ANNUAL PROGRESS REPORT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-01:	Vulnerability of Man to Biological Warfare
Study No. -00-09:	Influence of Irradiation on Infectious Diseases.
Reporting Installation:	U. S. Army Medical Unit Fort Detrick, Maryland
Divisions:	Pathology, Radiology and Virology
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Authors:	Charles C. Berdjis, Lt. Colonel, MC (Sect. I and II) Nelson R. Blemly, Lt. Colonel, MC (Sect. I) Robert W. McKinney, Major, MSC (Sect. I) Fletcher A. Reynolds, Captain, VC (Sect. I and II) Robert F. Jaeger (Sect. I)
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-01: Vulnerability of Man to Biological Warfare

Study No. -00-09: Influence of Irradiation on Infectious Diseases.

Section I. The Influence of Total-body X-irradiation on Infection with Venezuelan Equine Encephalomyelitis Viruses.

Reporting Installation: U. S. Army Medical Unit
Fort Detrick, Maryland

Divisions: Pathology, Radiology and Virology

Period Covered by Report: 1 July 1963 to 30 June 1964

Professional Authors: Charles C. Berdjis, Lt. Colonel, MC (Sect. I and II)
Nelson R. Blemly, Lt. Colonel, MC (Sect. I)
Robert W. McKinney, Major, MSC (Sect. I)
Fletcher A. Reynolds, Captain, VC (Sect. I and II)
Robert F. Jaeger (Sect. I)

Reports Control Symbol: RCS-MEDDH-288

Security Classification: UNCLASSIFIED

A series of experiments was conducted to evaluate the influence of total-body x-irradiation on infection with 2 strains of Venezuelan equine encephalomyelitis (VEE) virus (Trinidad and its attenuated strain TC-83).

The initial experiments were designed to attempt to establish the time parameters in which 200r or 500r might be expected to influance VEE infection in mice. Mice were administered 500r over a period ranging from 8 days before to 4 days after infection with approximately 10^3 $MIPLD_{50}$ Trinidad VEE. Except for those animals irradiated 8 hours before infection, mean days of death in remaining groups did not differ significantly from the nonirradiated controls. In a similar experiment with attenuated strain mice were challenged with virulent VEE 14 days after TC-83 administration; the pattern of death varied in the animals infected shortly before or shortly after irradiation. This resembled the "breakthrough" phenomenon observed on tissue culture neutralization test procedure. Maximum suppression of immunity occurred in the group irradiated one day before infection.

In similar experiments with 200r no effect of irradiation was observed.

In another study to determine whether irradiation altered the susceptibility of mice to infections with VEE virus, it appeared that irradiation prior to infection interfered with or altered the susceptibility of the mouse to infection.

It seemed most likely that the observed effect was due to altered capacity of irradiated cells to support virus replication and, depending on the duration of these effects, the immunological response to infection.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-01: Vulnerability of Man to Biological Warfare

Study No. -00-09: Influence of Irradiation on Infectious Diseases.

Section I. The Influence of Total-body X-irradiation on Infection with Venezuelan Equine Encephalomyelitis Viruses

Description:

To investigate the modifications of infectious diseases by x-ray irradiation.

To study the immunological responses and its possible impairment when irradiation is combined with infection.

Progress:

In these preliminary studies, particular emphasis was placed upon the time interval between radiation and infection and the effects of radiation on immunological responses. According to previous observations, irradiation might modify significantly the course of infectious diseases and infection might complicate the outcome of radiation sickness.

A series of experiments was conducted in this laboratory to evaluate the effect of x-irradiation on Venezuelan equine encephalomyelitis (VEE) infection. In these studies mice were employed to evaluate the influence of irradiation on infection with two strains of VEE virus: unmodified Trinidad strain and an attenuated strain of the same virus (TC-83). CD-1 strain, albino Swiss mice, 3-4 weeks of age were employed.

The unmodified Trinidad strain is uniformly lethal for all mice given an infecting dose, whereas its attenuated counterpart induces an inapparent infection as well as immunity to subsequent challenge with the parent virus. Mice recovered from infection with the attenuated virus have withstood challenge with up to 10^9 $MIPLD_{50}$ of unmodified virus.

X-irradiation was accomplished as a single whole-body exposure employing a 1 MEV, 3 MA unit at a distance of 100 cm and a rate of 55r per minute.

Experiment I was designed to try to establish the time parameters in which sublethal irradiation (500r) might be expected to influence the infection with either virus. Groups of mice were administered 500r over a period ranging from 8 days before to 4 days after infection with approximately 10^3 $MIPLD_{50}$ of Trinidad virus or 10^3 $MIPID_{50}$ of its attenuated strain. Table I shows

TABLE I. EFFECT OF X-IRRADIATION (TOTAL-BODY, 500r) ON VEE INFECTED MICE. DISTRIBUTION OF THE ANIMALS IN THE EXPERIMENTS.

GROUPS	VEE		OTHERS
	Trinidad	Attenuated (TC-83)	
I, Controls:			
1. Irradiation alone			20
2. Trinidad alone	20		
3. Attenuated VEE alone		20	
4. Unmodified			20
II, Irradiation + Infection (Simultaneously)	20	20	
III, Infection before Irradiation:			
1. - 8 hr.	20	20	
2. - 1 day	20	20	
3. - 2 days	20	20	
4. - 4 days	20	20	
IV, Infection after Irradiation:			
1. + 8 hr.	20	20	
2. + 1 day	20	20	
3. + 2 days	20	20	
4. + 4 days	20	20	
5. + 8 days	20	20	

the distribution of the mice in the experiments and Table II illustrates cumulative deaths by day post-infection and mean days of death. From analysis of these data, it appears that 500r did not significantly change the mortality rate of Trinidad infected mice. Except for those animals irradiated 8 hours after infection, the mean days of deaths in remaining groups did not differ significantly from the non-irradiated controls. Another observation recorded was early deaths which occurred within the -1 to +2-day groups: this was possibly related to a shortened incubation period as seen in bacterial infections.

Additional data were obtained with attenuated strain and summarized in Table III. Methods, procedures and distribution of the mice were the same as in the previous experiment (Table I). Evidence of infection was based on survival of the animals following challenge with virulent virus 14 days after inoculation of the attenuated strain.

Table III illustrates the cumulative deaths by days post-infection with the per cent surviving or immune. Except in the group irradiated one day before infection the pattern of death and/or day of first death varied from that usually observed with this type procedure. This was particularly true for the -1 day and -8-hour, simultaneous, and +8-hour groups. The pattern of death resembled the so-called "breakthrough" phenomenon observed in tissue culture neutralization test procedures. This is the first time we have observed this type response following attenuated virus and challenge procedure and will require further investigation.

Maximum suppression of immunity occurred in the group irradiated one day before infection. However, since development of immunity is dependent upon virus replication to produce sufficient antigenic mass to result in immunization, additional studies are indicated to define whether the influence is on that of virus replication or on the immune response or perhaps a combination of the two (see experiment III).

In experiment II similar procedures to those just described were performed employing 200r as the irradiation dose. The results may be summarized briefly, to wit, no effect of irradiation was observed.

TABLE II. PATTERN OF DEATH IN MICE ADMINISTEPED 500r X-IRRADIATION AND INFECTED WITH TRINIDAD STRAIN VEE VIRUS

TIME OF IRRADIATION RELATIVE TO INFECTION	CUMULATIVE DEATHS BY DAY POSTINOCULATION								MEAN DAY OF DEATH
	3	4	5	6	7	8	9	11	
Controls			10	18	20				5.6
-4 da[a]			8	11	19			20	6.25
-2 da			3	10	20				6.3
-1 da		2	9	16	18	19			.0
-8 hr		2	4	9	10	20			6.7
0	1	2	10	20					5.4
+8 hr[b]	1	4	13	19					5.1
+1 da		5	8	18	19				5.4
+2 da	1	2	9	16	18				5.4
+4 da		1	6	16	20				6.0
+8 da			5	9	18	19	20		6.4

a. Minus sign denotes irradiation after infection.

b. Plus sign denotes irradiation before infection.

TABLE III. PATTERN OF DEATH FOLLOWING CHALLENGE OF MICE ADMINISTERED 500r X-IRRADIATION AND INFECTED WITH ATTENUATED VEE VIRUS

TIME OF IRRADIATION RELATIVE TO INFECTION[a]	CUMULATIVE DEATHS BY DAY POSTCHALLENGE[b]													PER CENT IMMUNE
	4	5	6	7	8	9	10	11	12	13	14	15	16	
Controls														100
-4 da[c]			2											90
-2 da	1		3											85
-1 da					2					4	5			75
-8 hr							1			3	4		6	70
0					1	2			4	6				70
+8 hr[c]					2					3	4	6		70
+1 da		1	3	8	12	13								30
+2 da						1	2	3						85
+4 da														100
+8 da														100

a. Infected with Ca. 10^3 guinea pig intraperitoneal immunizing doses $_{50}$ of attenuated VEE virus.

b. Challenged with Ca. 10^3 $MIPLD_{50}$ Trinidad strain virus 14 days after original infection.

c. Minus denotes irradiation after infection, plus denotes re.

A comparison of the effects of 200r and 500r on attenuated VEE virus is presented in Figure 1. Each block represents the mortality rate of the irradiated mice following challenge with virulent virus 14 days after inoculation of the attenuated strain. Five hundred roentgens appeared to interfere with the mechanisms of defense and/or immunological responses, whereas 200r did not. Whether this difference is related to the reported detrimental effect of large doses and so-called beneficial effects of small doses, is not known; further studies will be needed to elucidate this question. Nontheless, this is in agreement with the findings of other authors in bacterial infections.

Experiment III. Because development of immunity with the attenuated virus is dependent on the replication of virus, it was of interest to determine whether irradiation altered the susceptibility of mice to infection with VEE virus. Mice were irradiated with 500r, 8 and 24 hours before, simultaneously with, and 8 and 24 hours after infection with unmodified Trinidad virus. The unmodified virus was titrated in serial 10-fold dilutions in each of these groups employing 10 animals per dilution. The results are presented in Table IV in which the LD_{50} in each group and the difference in logs from the control are given.

TABLE IV. TITRATION OF TRINIDAD STRAIN VEE VIRUS IN X-IRRADIATED MICE

TIME OF IRRADIATION RELATIVE TO INFECTION	VIRUS TITER[a]	DIFFERENCE FROM CONTROLS, LOG 10
Controls	10.5	-
-24 hr[b]	9.9	0.6
-8 hr	9.8	0.6
Simultaneous	9.8	0.7
+8 hr[2]	9.4	1.1
+24 hr	7.4	3.1

a. Virus titer expressed as $\log_{10}$/0.3 ml.

b. Minus denotes irradiation after. Plus denotes irradiation before.

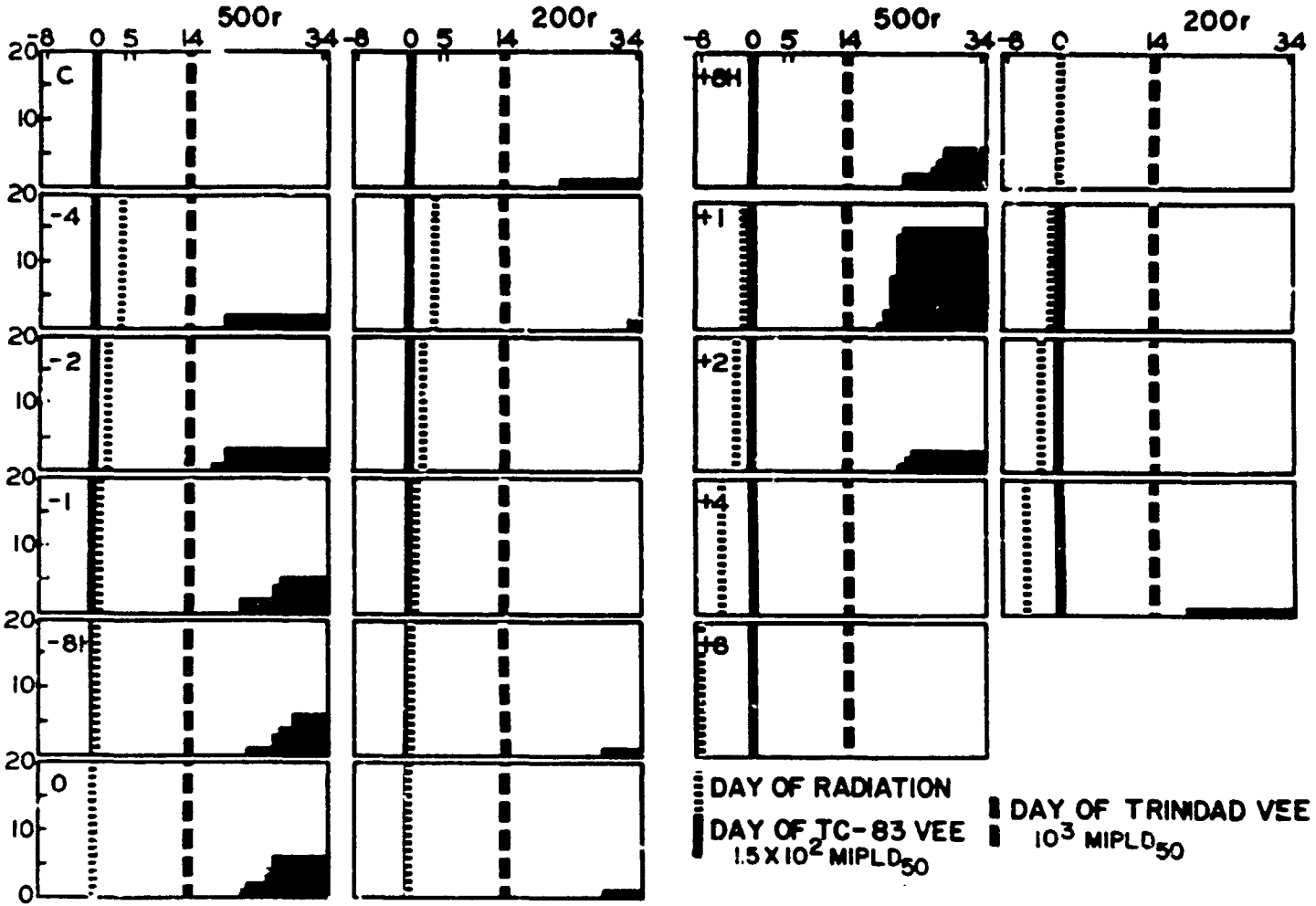


FIGURE I. COMPARATIVE EFFECTS OF 500r AND 200r ON MICE INFECTED WITH ATTENUATED VEE VIRUS AND CHALLENGED 14 DAYS LATER WITH UNMODIFIED VEE VIRUS.

The virus titered $10^{10.5}$ LD_{50} per 0.3 ml in the control group. The titers obtained in the controls and those animals irradiated simultaneously or after infection did not differ significantly. In contrast from the results obtained with animals irradiated 8 and 24 hours before infection it appeared that irradiation prior to infection with VEE virus, interferes with or alters the susceptibility of the mouse to infection with this agent. These results tend to support those obtained in the first experiment with attenuated virus.

It seems most likely that the observed effects are due to the altered capacity of irradiated cells to support virus replication and, depending on the duration of these effects, the immunologic response to infection. As employed, "virus replication" includes all phases of the cycle of virus infection. In addition, irradiation might induce or activate nonspecific humoral defense mechanisms which alter the infectious process.

Experiment IV. Since a direct effect on a cycle of virus replication seems the most likely of the proposed explanation, studies were initiated to determine the effect of irradiation on virus replication.

The approach employed involved the serial sacrifice of mice irradiated and infected as in the previous study. Three groups of mice were used in the initial study: those without irradiation, those irradiated simultaneously with infection, and those irradiated 24 hours before infection. These groups were selected since the latter particularly showed the most marked effect of irradiation on the infection.

For the purposes of estimating virus replication the liver of each mouse was removed and titrated. The liver was selected because of its size and ease of harvest and particularly because previous studies in our laboratory have shown this tissue to contain relatively the same quantities of virus as any other organ in mice infected with VEE virus.

The results of the titration in the three groups do not agree in total with those just presented (Experiment III). The titration in the controls and in the group irradiated simultaneously with infection were almost identical with those presented in the previous experiment. However, in the group irradiated 24 hours before infection, the difference between the controls and this group titer although significant, was 1.5 logs as compared to 3.1 logs obtained in the previous experiment. This failure to confirm the early results may be a technical error and will require repetition and further investigation.

Fluorescent antibody evaluation of VEE virus:

In a few animals sacrificed moribund in the first experiment, brain and visceral organs were frozen in liquid nitrogen for fluorescent antibody techniques. In addition, from experiment IV (serial sacrifice study) representative sections of liver and the following organs were also frozen

for virus detection by the same techniques: brain, spleen, kidney, heart and occasionally gonads or other tissues.

Fluorescent particles compatible with VEE virus (both modified and attenuated) were detected in the brains, livers and spleens of both irradiated and non-irradiated infected mice while none were seen in the controls. Figure 2 illustrates these fluorescent particles in brain of a VEE infected mouse, +8 hour-group.

Summary and Conclusions:

Several experiments were conducted to study the influence of irradiation on VEE infection and on immune responses. Mice were infected with Trinidad VEE virus or its attenuated strain. From the analysis of the data presented in these studies, the following conclusions are reached:

1. Irradiation influences VEE infection as it does most bacterial infections.

2. 500r did not significantly change the mortality rate of Trinidad VEE infected mice; a shortened incubation period for groups irradiated shortly before and after infection was the only pertinent finding.

3. In mice infected with the attenuated strain and challenged 14 days later with virulent VEE, the pattern of death varied from the controls and resembled the "breakthrough" phenomenon of neutralization test in tissue culture.

4. Immunity is maximally depressed in the group irradiated one day before infection.

5. 500r appears to interfere with the immunological responses, whereas 200r did not significantly alter these mechanisms.

6. Irradiation prior to infection interferes with or alters the susceptibility of the mouse to infection.

7. The observed effects are possibly due to an altered capacity of irradiated cells to support virus replication and/or immunological response to infection.

8. By fluorescent antibody techniques fluorescent particles are found in brain, spleen and liver of the VEE infected pre- or post-irradiated mice.

Publications:

None.

Presentation:

Presented on AFEB Commission on Irradiation and Infection, WRAIR, 1 May 1964.

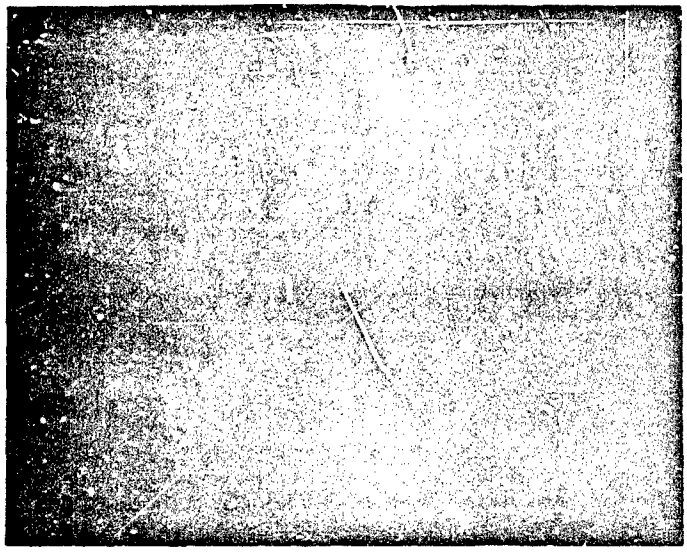


FIGURE 2. BRAIN OF A PRE-IRRADIATED VEE INFECTED MOUSE SHOWING FLUORESCENT PARTICLES WITH FLUORESCENT ANTIBODY TECHNIQUE.

ABSTRACT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-01: Vulnerability of Man to Biological Warfare

Study No. -00-09: Influence of Irradiation on Infectious Diseases

Section II. Pathologic Report. Histopathology of the Organs of Pre- or Postirradiated - Venezuelan Equine Encephalomyelitis Infected Mice.

Reporting Installation: U. S. Army Medical Unit
Fort Detrick, Maryland

Division: Pathology

Period Covered by Report: 1 July 1963 to 30 June 1964

Professional Authors: Charles C. Berdjis, Lt. Colonel, MC
Fletcher A. Reynolds, Captain, VC

Reports Control Symbol: RCS-MEDDH-228

Security Classification: UNCLASSIFIED

Mice randomly selected from each experimental group presented in Section I of this report were autopsied for histopathologic evaluation. The irradiated animals developed acute irradiation syndrome with generalized hemorrhage, and marked depletion of lymphoid and hematopoietic organs. Mice infected with Venezuelan equine encephalomyelitis (VEE) presented similar lesions with added meningoencephalitis.

Irradiation combined with VEE induced widespread necrotizing hemorrhagic lesions in most organs except brain. Occasionally brain showed a minimal perivascular cuffing or glial reaction. Thymus was maximally depleted in these animals.

Inhibition of CNS lesions in irradiated VEE infected mice suggests a possible blockage of immunological responses by irradiation.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-01: Vulnerability of Man to Biological Warfare

Study No. -00-09: Influence of Irradiation on Infectious Diseases

Section II. Pathologic Report. Histopathology of the Organs of Pre- or Postirradiated - Venezuelan Equine Encephalomyelitis Infected Mice.

Description:

To investigate the modifications of infectious diseases by x-r irradiation.

To study the immunological responses and its possible impairment when irradiation is combined with infection.

Progress:

From a histopathologic evaluation of the mouse organs in the present study and earlier reports, and a comparative study in guinea pigs (J Inf Dis 109: 62, 1961), it appears that: (a) the effects of irradiation and VEE infection are comparable, (b) VEE virus and x-rays exert a selective effect upon the lymphatic organs and bone marrow, and (c) both injure, incapacitate or destroy the lymphoid and hematopoietic cells.

In each experimental group of the initial experiments described in the Section I of this report, 3 or more mice were randomly selected for histopathologic evaluation. All animals were autopsied and representative sections of brain and visceral organs were fixed in 10% formalin, embedded in paraffin and stained with H & E for routine examination. In addition, specific stains such as PAS, trichrome, and luxol fast blue in combination with crystal violet were employed in selected sections.

For clarity this histopathologic report is divided in 3 parts: (a) x-irradiation, (b) VEE infection, (c) VEE infection and x-irradiation combined.

X-irradiation alone showed in mice the classical picture of acute irradiation syndrome characterized by hemorrhagic diathesis or extensive hemorrhage in the organs and partial destruction or depletion of the lymphoid and hematopoietic tissues (Figures 2, 3 and 5).

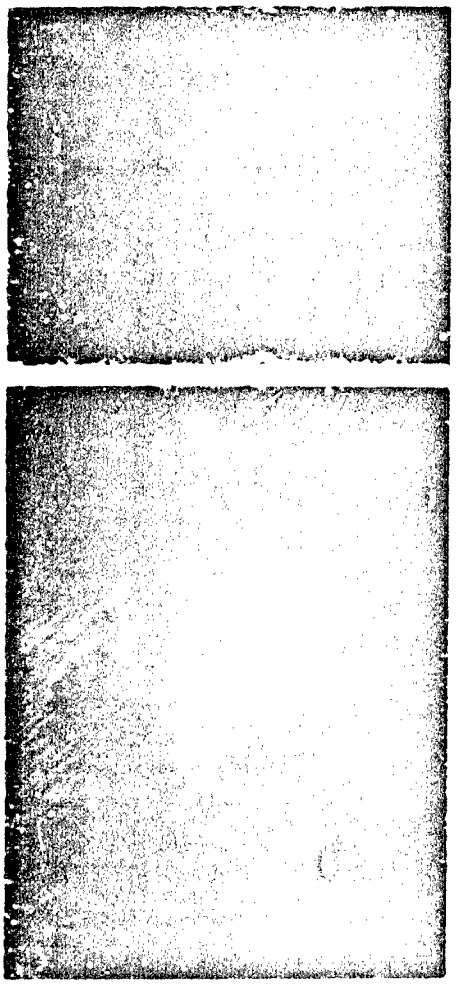


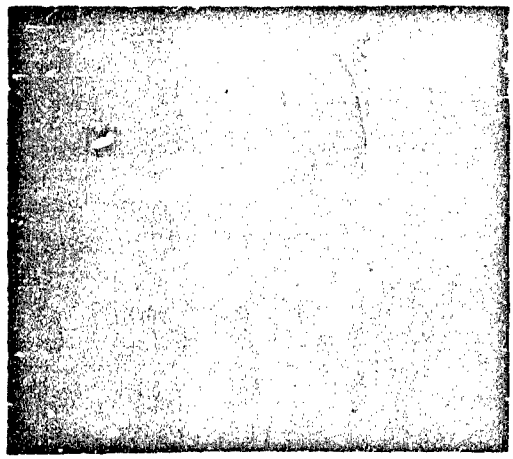
FIGURE 1. A & B: BRAIN IN VEE-INFECTED MICE: MENINGOENCEPHALITIS WITH EXTENSIVE PERIVASCULAR CUFFING & DIFFUSE GLIOSIS.

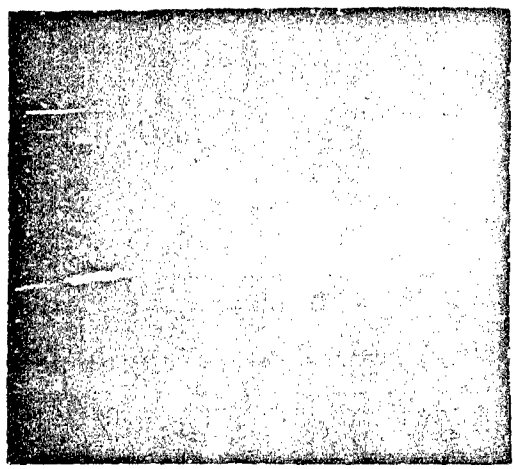
FIGURE 2. LYMPH NODES: A: X-IRRADIATION: DEPLETION OF THE LYMPHOID ELEMENTS & DISORDER IN THE ARCHITECTURAL PATTERN.

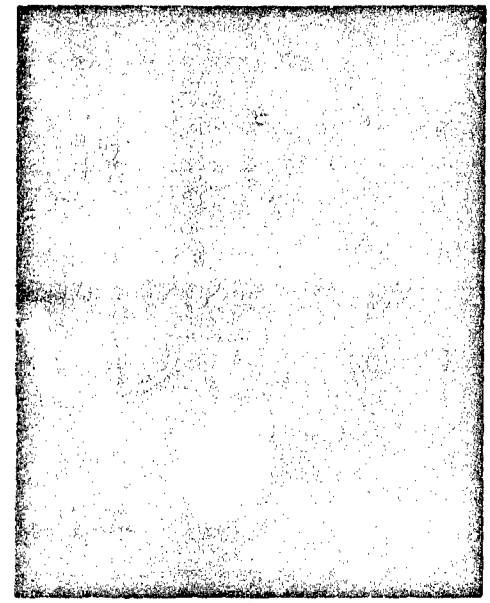
B: VEE-INFECTION: SIMILAR TO "A" SHOWING LYMPHOID DEPLETION & ARCHITECTURAL DISORDER.

C: VEE-IRRADIATION: GREATER DEGREE OF DEPLETION & MASSIVE LYMPHOID DESTRUCTION ASSOCIATED WITH DIFFUSE NECROSIS.

3A







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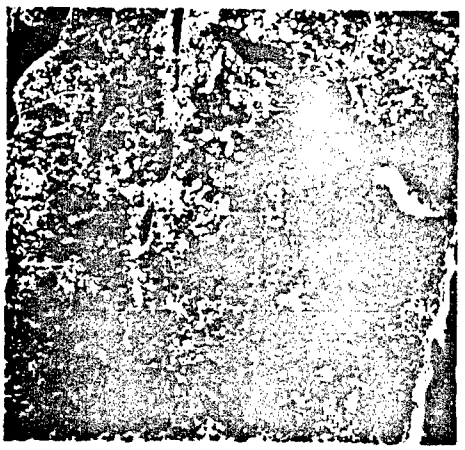
3B

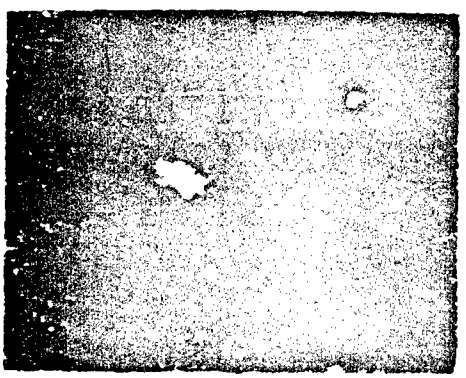
FIGURE 3. SPLEEN: A: X-IRRADIATION: LYMPHOID DEPLETION & ARCHITECTURAL DISORDER.

B: VEE + X-IRRADIATION: GREATER DEGREE OF LYMPHOID DEPLETION WITH DIFFUSE NECROSIS & HEMORRHAGE

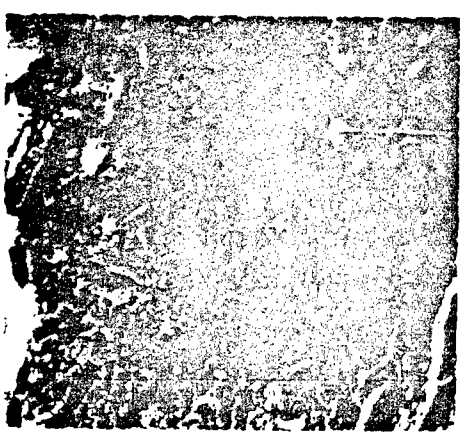
FIGURE 4. LIVER: FOCAL HEPATITIS IN A PRE-IRRADIATED VEE-INFECTED MOUSE.



5A



5B



5C

FIGURE 5. BONE MARROW: A: X-IRRADIATION: DEPLETION OF THE HEMATOPOIETIC ELEMENTS.

B: VEE INFECTION: DEPLETION OF THE HEMATOPOIETIC ELEMENTS & FOCAL NECROSIS.

C: VEE+IRRADIATION: MARKED DEPLETION & DISORGANIZATION OF THE HEMATOPOIETIC ELEMENTS WITH DIFFUSE NECROSIS.

VEE infection created in mice a mixed syndrome (visceral and cerebral) due to its pantropic nature. Visceral changes were similar to irradiation damage, i.e., depletion and partial destruction of the lymphoid and hematopoietic organs with focal hemorrhages and/or necrosis (Figures 2-5). Brain was invariably affected showing mild to marked meningoencephalitis with perivascular cuffing and focal or diffuse gliosis (Figure 1).

Thymus was depleted or partially destroyed. This was in contrast with guinea pig thymus which resisted VEE infection while it was depleted by irradiation.

VEE infection combined with irradiation, whether simultaneous or separate, extensively destroyed the lymphoid and hematopoietic organs (Figures 2-5). There was no significant difference between pre- or postirradiated infection. Widespread necrotizing hemorrhagic lesions were observed in most instances which destroyed sensitive organs including thymus. In some animals, however, a generalized hemorrhagic diathesis dominated the picture.

Although thymus was maximally destroyed by this combination, brain was much less if at all damaged. There appeared to be a predilection for the visceral organs with exclusion of an effect on the CNS. Irradiation seemed to disorganize the pantropism of VEE virus by inhibiting its so-called neurotropism. This finding suggests a possible block of immunological responses by irradiation and supports the data presented by Berge et al. (J Immunol 87:509, 1961) in modification of CNS response to VEE by immune serum. These authors attributed the histologic variation of CNS to interaction between VEE viral antigen and antibody. Our finding also confirms the data presented in Section I of this report and those reported in the literature relative to the interference of irradiation on immunological responses.

Summary and Conclusions:

1. As reported previously in guinea pigs, the comparable detrimental effect of VEE infection and irradiation on lymphoid and hematopoietic organs in mice is confirmed.

2. Both viscerotropism and neurotropism are present in VEE-infected nonirradiated animals. This confirms the pantropism of VEE virus.

3. Irradiation appears to inhibit the neurotropism of VEE virus, possibly by blocking the immunological responses.

4. Irradiation combined with VEE creates a widespread necrotizing hemorrhagic lesion in some animals and hemorrhagic diathesis in others.

5. In contrast with guinea pigs thymus is affected in VEE-infected mice.

Publications:

None.

ANNUAL PROGRESS REPORT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401AC96-01:	Vulnerability of Man to Biological Warfare
Study No. -10-01:	Quantitation of Human Susceptibility to Infection With Aged Aerosols of Pasteurella Tularensis
Reporting Installation:	U.S. Army Medical Unit Fort Detrick, Maryland
Division:	Medical
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Author:	William D. Sawyer, Major, MC
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-01: Vulnerability of Man to Biological Warfare

Study No. -10-01: Quantitation of Human Susceptibility to Infection With Aged Aerosols of Pasteurella Tularensis

Reporting Installation: U.S. Army Medical Unit
Fort Detrick, Maryland

Division: Medical

Period Covered by Report: 1 July 1963 to 30 June 1964

Professional Author: William D. Sawyer, Major, MC

Reports Control Symbol: RCS-MEDDH-288

Security Classification: UNCLASSIFIED

No additional studies have been performed during the reporting period. One further study is planned, however, for May 1964. The previous data are being summarized and a manuscript is in preparation.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C6224C1A096-01: Vulnerability of Man to Biological Warfare

Study No. -10-01: Quantitation of Human Susceptibility to Infection With Aged Aerosols of Pasteurella Tularensis

Description:

To determine the infectivity of aged aerosols of P. tularensis for experimental animals and ultimately man.

Progress:

No additional studies have been performed during the reporting period. One further study is planned, however, for May 1964. The previous data are being summarized and a manuscript is in preparation.

Summary:

No additional studies have been performed during the reporting period. One further study is planned, however, for May 1964. The previous data are being summarized and a manuscript is in preparation.

Publications:

None

ANNUAL PROGRESS REPORT

Project No. 1C012501A030:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C012501A030-01:	Vulnerability of Man to Biological Warfare
Study No. -20-01:	The Mechanism of Death in Anthrax in the Guinea Pig
Reporting Installation:	U. S. Army Medical Unit Fort Detrick, Maryland
Division:	Pathology
Period Covered by Report:	1 July 1963 through 30 June 1964
Professional Author:	Charles C. Berdjis, Lt. Colonel, MC
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project No. 1C012501A030: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C012501A030-01: Vulnerability of Man to Biological Warfare

Study No. -20-01: The Mechanism of Death in Anthrax in the Guinea Pig

Reporting Installation: U. S. Army Medical Unit
Fort Detrick, Maryland

Division: Pathology

Period Covered by Report: 1 July 1963 through 30 June 1964

Professional Author: Charles C. Berdjis, Lt. Colonel, MC

Reports Control Symbol: RCS-MEDDH-288

Security Classification: UNCLASSIFIED

Groups of young guinea pigs were infected with Venezuelan equine encephalomyelitis virus (both Trinidad and attenuated strains), tularemia or anthrax organisms and serially sacrificed. By a series of specific stains, histochemistry of liver cells was investigated in these animals. In the sections stained with Pyronin G or tetrazo method for protein, many liver cells in the infected animals were pyroninophilic and contained fat particles as early as day 1 postinfection. Controls did not show any significant modifications.

Histochemistry of liver cells in virus- and bacteria-infected guinea pigs showed that the degenerative changes were associated with the variations of lipid and protein content of the hepatic cells.

BODY OF REPORT

Project No. 1C012501A030: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C012501A030-01: Vulnerability of Man to Biological Warfare

Study No. -20-01: The Mechanism of Death in Anthrax in the Guinea Pig

Description:

T study the pathogenesis in guinea pigs of infection with Bacillus anthracis.

Progress:

When hepatic cells are injured by infectious processes a series of important histophysiopathologic changes occur in liver, some of which are demonstrable with the microscope.

It was important to establish if these changes are purely degenerative or associated with the variations of lipid and protein content of the hepatic cells.

The previous study (FY 1963 Annual Report) dealt with degenerative processes and disturbance of fat metabolism in bacterial infections.

In this report the emphasis is placed upon the histochemistry of liver cells in virus- and bacteria-infected guinea pigs.

Employing materials and methods previously described, 180 young guinea pigs, Hartley strain, weighing 400 gm on the average, were exposed to various infectious agents. Table I shows the distribution, dose and nature of infectious agents and routes of administration.

Two to 4 animals from each group were sacrificed daily for 5 days, and representative sections of each group were fixed for histopathologic evaluation. Both frozen and paraffin-embedded sections were prepared from formalin-fixed liver sections. Hematoxylin and Eosin (H & E) stain was used for routine histologic evaluation. The following stains were used for specific purposes: Periodic Acid Schiff with and without diastase digestion, Pyronin G, tetrazo method for protein, Brown and Brenn for bacteria, Oil red O and Sudan black B for lipids.

Liver plays a predominant role in carbohydrate metabolism, as well as a central role in the metabolism of neutral fats, phospholipids and cholesterol. In the present study by histochemical reactions, the morphological

variations of protein, phospholipids and neutral fats were investigated.

Varying degrees of damage to hepatic cells were observed in all experimental groups during the 5 days of the study. In addition, the variations of protein and phospholipids in hepatic cells and disturbance of fat metabolism engendered by infectious diseases were present in both virus- and bacteria-infected groups.

In the sections stained with Pyronin G or tetrazo method, many liver cells in the infected animals were pyroninophilic and contained fat particles as early as day one.

Histochemistry of liver cells in virus- and bacteria-infected guinea pigs showed that the degenerative changes are closely associated with the variations of protein and lipid content of the hepatic cells.

TABLE I. DISTRIBUTION OF GUINEA PIGS, INFECTIOUS AGENTS AND ROUTES OF INOCULATION (30 ANIMALS PER GROUP).

GROUP	INFECTIOUS AGENTS		DOSE	ROUTE OF ADMINISTRATION
	Gen. Name	Strain		
I	VEE[a/]	Trinidad	10^3GPIPID$_{50}$	Intraperitoneal
II	VEE	Attenuated (TC-82)	10^3GPIPID$_{50}$	Intraperitoneal
III	*B. anthracis*	V1b-189	5 x 10^3 spores	Subcutaneous
IV	*B. anthracis*	V1b-189	5 x 10^4 spores	Respiratory
V	*P. tularensis*	SCHU-S4	20 cells	Subcutaneous
VI	None	None	None	None

a. Venezuelan equine encephalomyelitis virus.

Summary:

Guinea pigs were infected with Venezuelan equine encephalomyelitis virus, *B. anthracis* spores or *P. tularensis* bacilli and serially sacrificed.

Specific stains such as Pyronin G or tetrazo method for protein showed that many liver cells in the infected animals were pyroninophilic and contained fat particles.

Histochemistry of liver cells in virus- and bacteria-infected guinea pigs showed that the degenerative processes were associated with the variations of protein and lipid.

Publications:

None.

ANNUAL PROGRESS REPORT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-01:	Vulnerability of Man to Biological Warfare
Study No. -20-02:	The Effect of Egg Yolk and Various Phosphatides Upon the Susceptibility of the Rat to Anthrax
Reporting Installation:	U. S. Army Medical Unit Fort Detrick, Maryland
Division:	Medical
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Author:	William D. Sawyer, Major, MC
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-01:	Vulnerability of Man to Biological Warfare
Study No. -20-02:	The Effect of Egg Yolk and Various Phosphatides Upon the Susceptibility of the Rat to Anthrax
Reporting Installation:	U.S. Army Medical Unit Fort Detrick, Maryland
Division:	Medical
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Author:	William D. Sawyer, Major, MC
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

No additional studies have been performed during the reporting period and none are planned for the immediate future. A manuscript is in preparation.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-01: Vulnerability of Man to Biological Warfare

Study No. -20-02: The Effect of Egg Yolk and Various Phosphatides Upon the Susceptibility of the Rat to Anthrax

Description:

To study the effect of egg yolk and phospholipids as suspending menstrua for spores on the susceptibility of rats to B. anthracis infection.

Progress:

No additional studies have been performed during the reporting period and none are planned for the immediate future. A manuscript is in preparation.

Summary:

No additional studies have been performed during the reporting period and none are planned for the immediate future. A manuscript is in preparation.

Publications:

None

ANNUAL PROGRESS REPORT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-01:	Vulnerability of Man to Biological Warfare
Study No. -20-03:	The Effect of Protein Deficiency Upon the Susceptibility of the Rat to Anthrax
Reporting Installation:	U.S. Army Medical Unit Fort Detrick, Maryland
Division:	Physical Sciences Division
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Author:	Irving Gray, Colonel, MSC
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-01:	Vulnerability of Man to Biological Warfare
Study No. -20-03:	The Effect of Protein Deficiency Upon the Susceptibility of the Rat to Anthrax
Reporting Installation:	U.S. Army Medical Unit Fort Detrick, Maryland
Division:	Physical Sciences Division
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Author:	Irving Gray, Colonel, MSC
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

Because of the transfer of personnel to higher priority projects and out of this unit, no further work was carried out during this fiscal yeer.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-01: Vulnerability of Man to Biological Warfare

Study No. -20-03: The Effect of Protein Deficiency Upon the Susceptibility of the Rat to Anthrax

Description:

To demonstrate the effect of specific amino acid deficiency on the resistance of the rat to Bacillus anthracis infection.

Progress:

Because of the transfer of personnel to higher priority projects and out of this unit, no further work was carried out during this fiscal year.

Publication:

1. Gray, I.: "The Effect of Protein Nutrition on Leukocyte Mobilization," Proc Soc Exp Biol Med; accepted for publication 1964.

Presentations:

None.

ANNUAL PROGRESS REPORT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-01:	Vulnerability of Man to Biological Warfare
Study No. -20-04:	The Effect of Anthrax on Cellular Metabolism
Reporting Installation:	U.S. Army Medical Unit Fort Detrick, Maryland
Division:	Physical Sciences Division
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Authors:	Irving Gray, Colonel, MSC Vernon P. Reed, SFC
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-01:	Vulnerability of Man to Biological Warfare
Study No. -20-04:	The Effect of Anthrax on Cellular Metabolism
Reporting Installation:	U.S. Army Medical Unit Fort Detrick, Maryland
Division:	Physical Sciences Division
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Authors:	Irving Gray, Colonel, MSC Vernon P. Reed, SFC
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

Because of higher priority work, there is no progress to report.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-01: Vulnerability of Man to Biological Warfare

Study No. -20-04: The Effect of Anthrax on Cellular Metabolism

Description:

To determine whether anthrax or toxin affects cellular metabolism and whether specific organs are affected.

Progress:

Because of higher priority work, there is no progress to report.

Summary and Conclusions:

None.

Publications:

None.

Presentations:

None.

ANNUAL PROGRESS REPORT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-01:	Vulnerability of Man to Biological Warfare
Study No. -20-05:	Biophysical Studies of *Bacillus anthracis* and its Metabolites
Reporting Installation:	U.S. Army Medical Unit Fort Detrick, Maryland
Division:	Physical Sciences Division
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Author:	Anne Buzzell, PhD
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-01: Vulnerability of Man to Biological Warfare

Study No. -20-05: Biophysical Studies of Bacillus anthracis and its Metabolites

Reporting Installation: U.S. Army Medical Unit
Fort Detrick, Maryland

Division: Physical Sciences Division

Period Covered by Report: 1 July 1963 to 30 June 1964

Professional Author: Anne Buzzell, PhD

Reports Control Symbol: RCS-MEDDH-288

Security Classification: UNCLASSIFIED

The objective of characterizing the biologically active metabolites of Bacillus anthracis requires that metabolites retain activity during any necessary concentration procedures. Concentration by ultrafiltration has proven to be most satisfactory. It allows virtually complete recovery of toxic activity simultaneous with removal of extraneous material that would interfere with analytic observation in the ultracentrifuge. Four sedimenting components are regularly observed with S^{20}_{w} of about 2.5s, 5s, 14s, and 19s. The 2.5s and 5s components are generally present in high and equal amounts. The faster pair are present in appreciable amounts only in very concentrated preparations and appear to be aggregates. The 2.5s component, never previously described, may be the primary toxic material.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-01: Vulnerability of Man to Biological Warfare

Study No. -20-05: Biophysical Studies of Bacillus anthracis and its Metabolites.

Description:

To characterize those macromolecules derived from Bacillus anthracis which have biological activity, such as those with lethal action or those which induce edema production, and to study such physical features of the organism which may have a bearing on the pathogenesis of the organism.

Progress:

In the 1963 report preliminary results were described concerning the effort to isolate and characterize anthrax toxin by methods which would minimize creation of artifacts. Work by others (Stanley & Smith: J Gen Microbiol 26: 49, 1961 and Smith & Stanley: J Gen Microbiol 29: 517, 1962) had indicated that the toxin consisted of 3 components, demonstrable after glass filtration of culture fluids. A "factor I," eluted from the filter by 0.3M, pH 9.7 bicarbonate buffer, had a high titer of guinea pig skin edema activity when recombined with filtrate which contained "factor II," or protective antigen. A "factor III," which could be found both in the filtrate and eluted from the filter was lethal for mice when recombined with factor II. The yields of factors I and III were extremely low and it seemed possible that glass filtration might have altered and destroyed much of the toxin.

The attempts to concentrate the toxin by dialysis against carbowax or to lyophilize it have been described. Activity of the lyophilized toxins was too low to be useful. The activity of carbowaxed toxin was high but small molecular weight components of the carbowax diffused through the dialysis tubing making the toxin preparations viscous and prevented optical observation of slowly sedimenting material. However, with a separation cell, it was found that the toxic activity sedimented no faster than the protective antigen component, whereas factors I and III are reported to sediment much faster.

Since the carbowax experiments showed that the toxin could be retained by dialysis tubing, ultrafiltration was employed to concentrate the toxin. All the data reported below are for toxin concentrated by ultrafiltration. The ultrafiltration technique, introduced by H. Tresselt, has been worked out in detail by A. Gaspar (Bacteriology Division, USAMU) and will be reported elsewhere in this Annual Report.

The first preparations were concentrated almost to dryness, with volume decreased 2000- to 7000-fold. The appearance of all the preparations in the analytical ultracentrifuge was qualitatively alike but varied quantitatively. Four sharply defined components, varying in amount, were always visible optically, with sedimentation constants, S^{20}_{w}, of about 2.5s, 5.0s, 14.0s, and 19.0s. The 2 fastest components probably correspond to factors I and III and the 5.0s to protective antigen. The 2.5s component has not been reported before. Lately preparations have been concentrated only 200- to 800-fold and back-washed from the tubing walls by allowing the tubing to soak in buffer solution prior to harvest. Much higher and more consistant yields of toxic activity (at least 50% of theoretical) have resulted and the sedimentation patterns have been quantitatively reproducible. With this material, the 2 slowest components occur in substantial and about equal amounts and the 2 fast components occur in amounts only barely visible optically. Therefore, it seems likely that the 14s and 19s components are aggregates of the 2 slower components. So far all preparations having high concentrations of 19s have had low concentrations of 5s, suggesting that 19s may be an aggregate of protective antigen. However, these relationships may have been fortuitous since material had been lost by adsorption on the dialysis tubing. If the 14s and 19s components arise from aggregations of the 2.5s and 5s components, they must either contain about 13 and 8 sub-units respectively*, or the sub-units must be folded more compactly in the aggregate. Folding seems the more plausible hypothesis, since intermediate size aggregates have never been observed. Also as indicated below, toxic activity surviving glass filtration is probably in an aggregated form.

Sedimentation data obtained with a separation cell (Yphantis & Waugh: J Phys Chem 60: 623 and 630, 1956) is given in Table I. It can be seen that in the absence of appreciable amounts of the 14s and 19s components, toxic activity migrates at a rate most similar to the 2.5s component. The data also indicates clearly that the 14s component carries toxic activity. It is, however, less clear whether the 19s component is toxic.

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*For spheres $S/S_n = \left(\frac{M}{nM}\right)^{2/3}$ where M is the molecular weight of the sub-units in the aggregate.

TABLE I. CENTRIFUGATION DATA WITH SEPARATION CELL

ULTRAFILTRATE RESIDUE (UFR) Conc. x crude toxin by vol.	CALCULATED FRACTION LEFT[a]		OBSERVED ACTIVITY LEFT[b] Time to death (Fischer rats)		ESTIMATED CONCENTRATION[c]			
	$Q_{2.5}$	$Q_{5.0}$	Control	Centrifuged	2.5s	5s	14s	19s
a 2000X	0.88	0.77	102(10X)	84(10X)	vh	vh	vl	l
b 2000X	0.86	0.74	57	97	h	h	m	l
b glass filt. 100X	0.83	0.67	52(10X)	>97(10X)	-	-	-	-
c 7000X Sephadex lyophilized 7000X	0.86	0.74	$\overline{125}$(5X)	3/3 lived (5X)	vh	vl	l	m
d 2300X	0.86	0.74	$\overline{83}$(23X)	$\overline{268}$(70X)	vh	l	m	vh
e 1200X	0.88	0.77	$\overline{101}$(60X)	2/3 lived (60X)	vh	l	l	vh
f 1200X	0.85	0.70	$\overline{84}$(60X)	$\overline{169}$(60X)	h	l	l	h
g 300X	0.82	0.67	$\overline{87}$	$\overline{98}$	m	m	vl	vl
h 900X	0.85	0.71	$\overline{87}$	$\overline{154}$	h	h	m	l
g+h 6 days 4°	0.84	0.70	$\overline{76}$(5X)	$\overline{75}$(5X)	m	m	l	vl
g+h 6 days 4°; 0.1M versene	0.82	0.65	$\overline{69}$(5X)	$\overline{67}$(5X)	m	m	vl	vl
g+h 6 days 4°; 10^{-3} Mg^{++}	0.83	0.67	$\overline{71}$(5X)	$\overline{72}$(5X)	m	m	l	vl
i 1000X	0.83	0.67	$\overline{58}$(5X), $\overline{206}$(1X)	$\overline{81}$(2.5X)	h	h	l	vl
i Sephadex 200X	0.78	0.59	$\overline{76}$(5X)	$\overline{92}$(5X)	-	-	-	-
j 850X	0.78	0.59	$\overline{95}$(2X)	$\overline{114}$(2X)	vh	vh	l	vl
j Sephadex UFR - 700X	0.79	0.59	$\overline{122}$(2X)	$\overline{130}$(2X)	m	m	-	-

a. Q_{14} and Q_{19} were 0.
b. Values with bar = averages for 3 rats. Concentration in ( ); when not stated = 1X. Typical dilution curve for crude toxin = 67(1X); 89(0.5X); 149(0.25X).
c. Based on areas under schlieren curves from photographs taken during the separation cell centrifugation.
h = high; m = medium; l = low; v = very.

The question remains whether both slow components are required for toxic action. Two components are required for toxic action in mice for preparations fractionated by glass filtration. However, suggestive evidence of damage to the 2.5s component has been obtained using preparations of glass filtered crude toxin. In the filtrates, concentrated by ammonium sulfate precipitation, the originally sharp 2.5s boundary became diffuse with much material sedimenting very slowly. By contrast the 5s boundary remained sharp. In a pH 9.7 sodium bicarbonate extract of the filter a similarly diffuse 2.5s component was found. It is intended to seek means other than glass filtration for separating the 2.5s and 5.0s components. Possibly they can be isolated by density gradient centrifugation.

Summary and Conclusions:

Highly active preparations concentrated by ultrafiltration contain 4 sedimenting components with S^{20}_{w} of about 2.5s, 5s, 14s, and 19s. The 14s and 19s components appear to be aggregates, possibly of the 2.5s and 5s components respectively. The 2.5s and 5s components are present in high and equal concentrations. The 5s component is probably the well known protective antigen. The 2.5s component has not been described before. Earlier procedures for producing the nontoxic fraction, protective antigen, regularly had a glass filtration step which may damage the 2.5s component. Results with a separation cell so far suggest that the 2.5s component may be the true toxin but unambiguous conclusions about the roles of the 2.5s and 5s components must await their isolation.

Publications:

None.

Presentations:

None.

ANNUAL PROGRESS REPORT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-01:	Vulnerability of Man to Biological Warfare
Study No. -40-01:	The Effect of Virus Infection on Host Cell Energy Metabolism
Reporting Installation:	U.S. Army Medical Unit Fort Detrick, Maryland
Division:	Physical Sciences Division
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Author:	Irving Gray, Colonel, MSC
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-01:	Vulnerability of Man to Biological Warfare
Study No. -40-01:	The Effect of Virus Infection on Host Cell Energy Metabolism
Reporting Installation:	U.S. Army Medical Unit Fort Detrick, Maryland
Division:	Physical Sciences Division
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Author:	Irving Gray, Colonel, MSC
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

Because of transfer of personnel and equipment to a higher priority study, this study has been held in abeyance.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-01: Vulnerability of Man to Biological Warfare

Study No. -40-01: The Effect of Virus Infection on Host Cell Energy Metabolism

Description:

To investigate the energy metabolism of animals that have been infected with Venezuelan equine encephalomyelitis.

Progress:

Because of transfer of personnel and equipment to a higher priority study, this study has been held in abeyance.

Publications:

None.

Presentations:

None.

ANNUAL PROGRESS REPORT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-01:	Vulnerability of Man to Biological Warfare
Study No. -40-02:	The Effect of Virus Infection on Host Cell Protein Metabolism
Reporting Installation:	U.S. Army Medical Unit Fort Detrick, Maryland
Division:	Physical Sciences Division
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Author:	Jerry R. Mohrig, 1st Lieutenant, MSC
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-01: Vulnerability of Man to Biological Warfare

Study No. -40-02: The Effect of Virus Infection on Host Cell Protein Metabolism

Reporting Installation: U.S. Army Medical Unit
Fort Detrick, Maryland

Division: Physical Sciences Division

Period Covered by Report: 1 July 1963 to 30 June 1964

Professional Author: Jerry K. Mohrig, 1st Lieutenant, MSC

Reports Control Symbol: RCS-MEDDH-288

Security Classification: UNCLASSIFIED

The effects of *in vivo* Venezuelan equine encephalomyelitis virus infection upon *in vitro* protein synthesis by host cell fractions has been studied. An improved system for this study of *in vitro* protein biosynthesis has been developed utilizing the ability of microsome and enzyme cell fractions to incorporate L-Leucine-C14 into protein as a measure of the host cell's ability to synthesize protein. No clear difference in *in vitro* protein biosynthesis is observed between infected and uninfected mouse brain microsome and enzyme preparations.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-01: Vulnerability of Man to Biological Warfare

Study No. -40-02: The Effect of Virus Infection on Host Cell Protein Metabolism

Description:

To demonstrate the effect of Venezuelan equine encephalomyelitis virus upon host cell protein synthesis.

Progress:

Brain and liver tissues from uninfected mice and those infected with Venezuelan equine encephalomyelitis (VEE) have been homogenized, and cell microsomes and soluble RNA and enzymes have been obtained by the use of preparative ultracentrifugation. An in vitro cell-free incubation system has been made containing an ATP regenerating system, GTP, $MgCl_2$, an amino acid mixture containing 18 amino acids but excluding L-Leucine, L-Leucine-C14, and enzymes precipitable at pH 5.2. The Mg concentration in the media used during the harvesting and storage of the cell microsome and enzyme fractions has been shown to be very important in the ability of these fractions to effect in vitro protein biosynthesis. The requirements for optimal L-Leucine-C14 incorporation into protein synthesized with mouse brain microsomes are remarkably similar to those previously shown by other workers with liver microsomes.

Preliminary work in this laboratory (Gray and French) indicated that a marked increase in in vitro protein synthesis occurs in brain microsomes obtained from mice sacrificed 2 to 3 days subsequent to infection with VEE. Results obtained in the same manner, but using the improved incorporation system and carefully controlled Mg ion concentrations, have shown that no significant change in L-Leucine-C14 incorporation into protein occurs in brain microsomes during the course of VEE virus infection when compared to controls. High virus titers consistently have been shown to be present in the tissues studied. Further work is being done using infected mouse liver microsomes to study the effects of VEE virus infection upon protein synthesis.

Current efforts are being directed toward relating the results obtained through L-Leucine-C14 incorporation studies with the physical characteristics of the microsomes. This work is being done in conjunction with Dr. Anne Buzzell. Sodium deoxycholate conversion of brain and liver microsomes has led to the isolation of ribosomes (ribonucleoprotein) which have been shown to exhibit in vitro protein synthesis. Preliminary sucrose density gradient ultracentrifugation

work has shown that the ribosomes obtained can be separated into fractions of different molecular weights. Physical changes in ribosomal appearance in VEE virus infection will be correlated with the protein synthetic ability of the microsomes from which they were derived. Electron microscopic studies of the isolated ribosomal material has been done. Attempts to relate VEE virus titers to the distribution of the virus in the mouse liver are being carried out by electron microscopic techniques.

Summary and Conclusions:

Improved in vitro cell-free protein synthesizing systems have been prepared from both mouse brain and liver tissues. These systems have been used to study the effect of VEE virus infection upon protein biosynthesis. Cell microsome and enzyme fractions from both infected and uninfected mouse brain have shown no clear differences in in vitro biosynthesis. Biophysical studies have been initiated to study the effects of VEE virus infection upon cell ribosomal material and to demonstrate the distribution of VEE virus in liver tissue.

Publications:

None.

Presentations:

None.

ANNUAL PROGRESS REPORT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-01:	Vulnerability of Man to Biological Warfare
Study No. -40-03:	Biophysical Studies of Venezuelan Equine Encephalomyelitis
Reporting Installation:	U.S. Army Medical Unit Fort Detrick, Maryland
Division:	Physical Sciences Division
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Author:	Section I and II Anne Buzzell, PhD
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-01: Vulnerability of Man to Biological Warfare

Study No. -40-03: Biophysical Studies of Venezuelan Equine Encephalomyelitis
Section I. Concentration of the Virus

Reporting Installation: U.S. Army Medical Unit
Fort Detrick, Maryland

Division: Physical Sciences Division

Period Covered by Report: 1 July 1963 to 30 June 1964

Professional Author: Anne Buzzell, PhD

Reports Control Symbol: RCS-MEDDH-288

Security Classification: UNCLASSIFIED

In order to determine if physical or chemical properties of the attenuated strain of Venezuelan equine encephalomyelitis virus may be related to its decreased virulence, concentrated and highly purified stocks of virus must first be prepared. Initial experiments indicated that ultracentrifugation techniques were potentially useful in concentrating the virus. Subsequently, experiments indicated that tissue debris, present in considerable quantity in guinea pig heart cell tissue culture fluids, interfered seriously with the concentration procedure used. Effort has also been focused on developing a rapid and sensitive assay for the virus particles. In this assay, viral particles are first concentrated by centrifugation or millipore filtration and then transferred to an electron microscope grid for counting. The early version of the method, reported in 1963, has been substantially modified making it easier, more reliable, and potentially much more sensitive. The possibility of its use for early diagnosis is now being investigated.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-01: Vulnerability of Man to Biological Warfare

Study No. -40-03: Biophysical Studies of Venezuelan Equine Encephalomyelitis
Section 1. Concentration of the Virus

Description:

To characterize the virus of Venezuelan equine encephalomyelitis in terms of its size, shape and chemical constitution. Further, with the techniques and equipment available, to demonstrate physical or chemical properties associated with the change in virulence of the Trinidad strain to the attenuated strain.

Progress:

In the 1963 report the difficulties encountered in concentrating and purifying the attenuated strain of Venezuelan equine encephalomyelitis were described in detail. The two principal difficulties were that the virus appears to be unstable even at 4C and the virus which is grown in guinea pig heart cell tissue cultures is heavily contaminated with tissue debris. To hasten the process of evolving a purification procedure, a rapid assay method was needed. An early version of such an assay method was also described in the 1963 report. Subsequently, the method has been considerably simplified and the quantitative reliability has been substantially improved. Means of extending the technique to such dilute viral suspensions as sera of infected individuals are being explored with a view to use of the technique for early diagnosis.

The assay is based on the method of Sharp (Proc Soc Exp Biol Med 70: 54, 1949) in which viral particles were centrifuged onto a block of agar, stripped from the agar by a collodion film, and then placed on an electron microscope grid for counting. The film was formed by letting a drop of collodion run over the agar which was then floated from the agar, virus-side down into distilled water. This procedure was found to be tedious and unreliable, for the small pieces of film were often lost.

The electron microscope assay method in its present form contains numerous improvements. A formvar film is now made by the widely used procedure of dipping a microscope slide into a formvar solution. When dry, the film is broken around the edge and floated off on water. A piece of "saran wrap," perforated by a hole slightly larger than an electron microscope grid, is stretched taut over a frame (described in the 1963 report) and touched down on the formvar film. This adheres to the saran and covers the hole. An agar block bearing virus particles

is pressed against the film through the hole. The frame is inverted and the agar drops off after a corner is loosened with a scalpel. The frame is then lowered over an electron microscope grid resting on saran wrap so that the formvar film is stretched over the grid and adheres to it. It was initially thought the formvar film might not make uniform contact with the agar and so miss some virus particles unless it were held rigid by a second block of agar placed underneath. Hence, the films were formerly made on agar by the relatively laborious procedure described before. It later became apparent that particles were unevenly distributed on the agar because of its surface heterogeneity. Since then Difco "Noble agar" has been substituted for "Bacto-agar" and even the simplified retrieval procedure described yields uniform particle distribution on the formvar.

In attempting to improve methods for concentrating viral particles prior to counting, it is evident that the sensitivity of the viral assay would be increased by lengthening the column of infected fluid and by decreasing the area on which the particles sediment. Column length is limited if particles are sedimented in the centrifuge but not if particles are deposited by filtration. The use of filtration for concentrating viral particles was first considered after it was discovered by Major Thomas J. Smith, Medical Division, USAMU, that virus particles would adhere to agar which was pressed against a millipore filter.

A filtration area of one cm^2 (about 10 grid areas) is the smallest attainable with a commercial high pressure filtration device. Since the field of a micrograph at 8000X magnification is 4×10^{-7} cm^2, 10 ml of an infected serum with 10^6 particles/ml (about 10^4 to 10^5 infectious units) would theoretically yield 4 particles per micrograph. At the maximum allowed pressure, 10 ml water takes 20 minutes to pass through a 10-20 mμ pore size filter and whole serum clogs such a filter. Therefore, preliminary separation of the virus from sera will probably be necessary and might be accomplished by ultracentrifuging the virus into a small volume of sucrose solution layered below the serum. A filtration device with a much smaller filter area, which should be feasible, would reduce the volume and infectivity level of serum required for virus detection. Virus identification probably cannot be done solely on the basis of morphology and may require the use of ferritin-labeled specific antisera.

Summary and Conclusions:

An assay method has been devised for detecting low concentrations of virus-like particles visible in the electron microscope. The particles are sedimented onto agar and then transferred to an electron microscope grid by stripping them from the agar with a performed formvar film. The particle count under the electron microscope gives a qunatitative measure of particle concentration in the original fluid. An earlier version of this assay method, described in the 1963 report, has been modified to make it much easier and more reliable. Furthermore, it has been found possible to substitute filtration for centrifugation as the means of concentrating the virus, since virus particles could easily be stripped from a millipore filter surface by an agar block. Using millipore filtration, it should be

possible to increase the sensitivity of the assay method manyfold. Therefore, the possibility is now being explored of adapting the procedure for diagnostic use in the early detection of viremia.

The new assay will also expedite the search for a better procedure for concentrating attenuated VEE virus and freeing it from the cell debris in guinea pig heart cell tissue culture fluids.

Publications:

None.

Presentations:

None.

ABSTRACT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-01: Vulnerability of Man to Biological Warfare

Study No. -40-03: Biophysical Studies of Venezuelan Equine Encephalomyelitis
Section II. Electron Microscope Study of the Infected Mouse Brain

Reporting Installation: U.S. Army Medical Unit
Fort Detrick, Maryland

Division: Physical Sciences Division

Period Covered by Report: 1 July 1963 to 30 June 1964

Professional Author: Anne Buzzell, PhD

Reports Control Symbol: RCS-MEDDH-288

Security Classification: UNCLASSIFIED

The electron microscopic investigation initiated in 1963 on the interaction of VEE virus with the host cell is continuing. The emphasis of the study has been shifted from infected brain to liver to allow collaboration with work being done by others on the protein synthetic ability of normal vs. infected hepatic cells.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-01: Vulnerability of Man to Biological Warfare

Study No. -40-03: Biophysical Studies of Venezuelan Equine Encephalomyelitis
Section II. Electron Microscope Study of the Infected Mouse Brain

Description:

To characterize the virus of Venezuelan equine encephalomyelitis in terms of its size, shape and chemical constitution. Further, with the techniques and equipment available, to demonstrate physical or chemical properties associated with the change in virulence of the Trinidad strain to the attenuated strain.

Progress:

In the 1963 report, preliminary experiments were reported of collaboration with Lt. Colonel C.C. Berdjis, Pathology Division, USAMU, on the electron microscope study of infected mouse brain infected with Venezuelan equine encephalomyelitis virus. Because of the absence of Col. Berdjis, it has been decided to shift temporarily the emphasis of the study to virus interaction with another host cell such as mouse liver. This will have the advantage of correlation with a study by Colonel Irving Gray and Lt. Jerry R. Mohrig, Physical Sciences Division, USAMU, on the effect of virus on the protein synthetic ability of microsomal fractions of hepatic cells. The importance of brain damage in infections with VEE and related viruses make eventual study of brain-virus interaction a desirable final goal. However, studies of Gray and Mohrig, (Study No. 01-40-02) recorded elsewhere in this annual report suggest that microsomal preparations obtained from normal and infected brain show no clear differences in protein synthetic ability. It is believed that failure to demonstrate differences may be due to localization of the virus in only certain types of brain cell. They are therefore planning to initiate studies of protein synthesis by microsomes using liver as the host cell system which may have a more uniform distribution of infectivity since it has less diversity of cell types than brain. Concurrent with the electron microscopic collaboration, it is intended to collaborate on a study of the physical properties of the microsomes using ultracentrifugation techniques. Preliminary separations of the ribosomal moieties have been made in sucrose density gradients for microsomal preparations from uninfected cells.

Summary and Conclusions:

Electron microscopic examination of mouse liver infected with VEE virus is under way in correlation with a study by others on the ability of infected hepatic cell microsomes to synthesize protein. A physical study of the isolated microsomes using centrifugation techniques has also been initiated.

Publications:

None.

Presentations:

None.

ANNUAL PROGRESS REPORT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-01:	Vulnerability of Man to Biological Warfare
Study No. -40-04:	Modification of Response of Macaca mulatta to Venezuelan Equine Encephalomyelitis by Antibody Administration
Reporting Installation:	U. S. Army Medical Unit Fort Detrick, Maryland
Division:	Pathology
Period Covered by Report:	1 July 1963 through 30 June 1964
Professional Author:	Donald H. Yost, Lt. Colonel, VC
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-01:	Vulnerability of Man to Biological Warfare
Study No. -40-04:	Modification of Response of Macaca mulatta to Venezuelan Equine Encephalomyelitis by Antibody Administration
Reporting Installation:	U. S. Army Medical Unit Fort Detrick, Maryland
Division:	Pathology
Period Covered by Report:	1 July 1963 through 30 June 1964.
Professional Author:	Donald H. Yost, Lt. Colonel, VC
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

No progress has been made during the reporting period and the study is being terminated.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-01: Vulnerability of Man to BW

Study No. -40-04: Modification of Response of *Macaca mulatta* to Venezuelan Equine Encephalomyelitis by Antibody Administration

Description:

To define the interaction between active infection with Venezuelan equine encephalomyelitis (VEE) virus in the rhesus monkey and VEE antibodies.

Progress:

Monkeys have not been available during this past year for the continuation of these investigations, due to commitments to other projects of a higher priority. There has been no progress during the reporting period and the study is being terminated.

Summary and Conclusions:

None.

Publications:

None.

ANNUAL PROGRESS REPORT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-01:	Vulnerability of Man to Biological Warfare
Study No. -40-05:	Role of Antibody in the Clinical Manifestations of Venezuelan Equine Encephalomyelitis Infection
Reporting Installation:	U. S. Army Medical Unit Fort Detrick, Maryland
Division:	Virology
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Authors:	Warren R. Brunton, Captain, VC Robert W. McKinney, Major, MSC David G. Crist, SP4, AMEDS
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project No. 1C 22401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C6224.1A0? -01: Vulnerability of Man to Biological Warfare

Study No. -49-05: Role of Antibody in the Clinical Manifestations of Venezuelan Equine Encephalomyelitis Infection

Reporting Installation: U. S. Army Medical Unit
Fort Detrick, Maryland

Division: Virology

Period Covered by Report: 1 July 1963 to 30 June 1964

Professional Authors: Warren R. Brunton, Captain, VC
Robert W. McKinney, Major, MSC
David G. Crist, SP4, AMEDS

Reports Control Symbol: RCS-MEDDH-288

Security Classification: UNCLASSIFIED

The administration of passive antibody has been shown to be effective in the modification of Venezuelan equine encephalomyelitis infection in monkeys and guinea pigs. The response of the animal was found to vary with the amount of passive antibody and its time of administration relative to inoculation of virus.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-01: Vulnerability of Man to Biological Warfare

Study No. -40-05: Role of Antibody in the Clinical Manifestations of Venezuelan Equine Encephalomyelitis Infection

Description:

To investigate the possible value of passive antibody in the treatment of Venezuelan equine encephalomyelitis virus infection.

Progress:

Initially monkeys were employed to study the influence of passive antibody on infection with Venezuelan equine encephalomyelitis (VEE) virus. To determine the longevity of passively administered antibody in monkeys, 3 rhesus monkeys were inoculated intravenously with homologous VEE immune serum having a hemagglutinating-inhibiting (HAI) titer of 1:2560 and a serum neutralization index (SNI) greater than 7.2 logs. The volume of serum administered was based on the animals' weight, i.e., 2.0, 4.0, or 8.0 ml/kg. Table I presents antibody titers observed in these animals on various days after administration of serum.

TABLE I. ANTIBODY TITERS IN MONKEYS FOLLOWING ADMINISTRATION OF HOMOLOGOUS VEE IMMUNE SERUM

MONKEY NO.	SERUM ml.	TEST	DAY AFTER INOCULATION OF SERUM 1	7	13	20	27	34	41
E-36	5.8	HAI[a]	40	20	<10	<10	[c]		
		SNI[b]	2.39	1.04	1.52	1.24			
CC-15	11.2	HAI	80	40	10	<10			
		SNI	6.98	2.85	≤1.63	0.64			
CC-10	21.6	HAI	160	80	40	40	20	10	10
		SNI	≥7.64	≥7.64	4.89	3.94	2.64	1.78	1.14

a. HAI - Expressed as reciprocal of titer.
b. SNI - Serum neutralization index expressed as $\log_{10}$.
c. Blank space denotes not done.

Following the establishment of the normal decay curve of homologous serum in monkeys, the work was extended to examine the influence of passive antibody on infection with VEE virus. In this experiment 4 monkeys were each inoculated subcutaneously with approximately 10^3 mouse intraperitoneal median lethal doses ($MIPLD_{50}$) of Trinidad strain virus. At intervals of 4, 8, and 24 hr following inoculation of virus, 3 ml of homologous VEE immune serum per kilogram body weight was administered subcutaneously to one of the animals. The fourth animal served as the virus control. The HAI titer of the serum was 1:2560 and the SNI was 7.0 $logs_{10}$.

Daily heparinized blood samples were inoculated into mice for determination of levels of viremia. Results of these assays are presented in Table II. Serology results appear in Table III.

TABLE II. TITERS[a] OF CIRCULATING VIRUS FOLLOWING INOCULATION OF VIRUS AND SERUM

MONKEY NO.	TIME SERUM ADMINISTERED AFTER VIRUS INOCULATION	$LOGS_{10}$ VIRUS RECOVERED BY DAY AFTER VIRUS INOCULATION						
		1	2	3	4	5	6	7
N-22	4 hr	0[b]	0	0	0	0	0	0
N-41	8 hr	0	0	0	0	0	0	0
N-13	24 hr	4.0[c]	0	0	0	0	0	0
M-46	Virus Control	$\geq$3.0	$\geq$4.0	2.9	0.2	0	0	0
A-19	Serum Control	0	0	0	0	0	0	0

a. Expressed as log_{10} of mouse intraperitoneal median lethal doses ($MIPLD_{50}$) per 0.3 ml.
b. 0 - Virus not recovered from undiluted blood.
c. This sample obtained immediately prior to inoculation of serum.

From the data in Table II it can be seen that administration of immune serum at 4 and 8 hr after inoculation of virus blocked the appearance of virus in the circulation. Similarly administration of serum at 24 hr resulted in clearing of the viremia. However, the results of serologic tests (Table III) show that the administration of serum as soon as 4 hr after virus inoculation did not obliterate the infection.

It is of interest that the rate and magnitude of antibody response in this animal, i.e., Monkey N-22, are below those observed in animals receiving serum at the 8-hr and 24-hr intervals. It is also noteworthy that the increase in antibody in this animal occurred at approximately 28 days; a time when the

TABLE III. ANTIBODY TITERS IN MONKEYS ADMINISTERED IMMUNE SERUM AT INTERVALS AFTER VEE VIRUS

MONKEY NO.	TIME SERUM ADMINISTERED AFTER VIRUS INOCULATION	TEST	ANTIBODY TITERS Day after virus inoculation 1	5	7	14	28	44
N-22	4 hr	HAI[a]	40	40	20	40	80	320
		SNI[b]	3.0	[c]	4.4	≤1.6	2.4	6.5
N-41	8 hr	HAI	20	20	20	1280	5120	2560
		SNI	1.7		2.5	≥6.5	≥6.5	
N-13	24 hr	HAI	40[d]	40	40	320	1280	2560
		SNI	3.1		2.7	≥6.5	≥6.5	
M-46	Virus Control	HAI	<10	10	80	640	1280	5120
	No Serum	SNI	≤0.6	1.35			6.5	
A-19	Serum Control	HAI	20	40	20	20	<10	<10
	No Virus	SNI	1.5		3.4	1.6	1.0	

a. HAI - expressed as reciprocal of titer.
b. SNI - expressed as $\log_{10}$.
c. Blank space denotes not done.
d. This sample only was obtained 24 hr later than others in the same category.

passive HAI antibody in the serum control had decreased to <1:10. Taken together these results suggest that infection in Monkey N-22 was of low magnitude and was manifested only after depletion of the passive antibody. In contrast, the rate and magnitude of antibody increase in the animals administered serum 8 and 24 hr after virus were similar to that observed in the virus control.

Although the number of animals was small, the study indicated that infection with VEE virus was modified by the administration of passive antibody. Because unmodified VEE virus infection is uniformly lethal in the nonimmune animal, guinea pigs were selected for further studies.

A study to determine the longevity of passively administered antibody in guinea pigs consisted of 2 trials employing animals of different weights. In the first trial, 250-350-gm guinea pigs received intraperitoneally (IP) varying amounts of homologous VEE immune guinea pig serum (HAI titer 1:1280). The immune serum in this and subsequent studies was obtained from guinea pigs which had recovered from infection with the nonlethal attenuated strain of VEE virus.

The mean reciprocal HAI titer of each group on various days following administration of serum is presented in Table IV. Due to technical

difficulties, 10 animals, 2 from group II and 4 each from groups III and IV, died following the first bleeding. Thus, the values after day 1 for the latter groups are derived from 2 animals each.

TABLE IV. HAI TITERS[a] OF GUINEA PIGS FOLLOWING ADMINISTRATION OF HOMOLOGOUS VEE SERUM

GROUP NO.	SERUM PER ANIMAL ml.	MEAN RECIPROCAL HAI TITER BY DAY AFTER SERUM ADMINISTRATION 1	6	12	18	25	32	40
I	1.0	20	8	7	6	5	[b]	
II	2.0	25	12	11	6	6		
III	4.0	73	40	30	20	10	5	
IV	8.0	160	120	60	40	30	10	5

a. For purposes of computation, sera with titers of less than 1:10 (the lowest dilution used in the HAI test) were assigned a value of 5.
b. Blank space denotes not done.

Because of difficulties in bleeding the smaller animals 350-450-gm guinea pigs were employed in the next experiment. Antibody titers for these animals appear in Table V.

TABLE V. HAI TITERS[a] OF GUINEA PIGS FOLLOWING ADMINISTRATION OF HOMOLOGOUS VEE IMMUNE SERUM

GROUP NO.	SERUM[b] PER ANIMAL ml.	MEAN RECIPROCAL HAI TITERS BY DAY AFTER SERUM ADMINISTRATION 1	6	12
Ia	1.0	6	5	5
IIa	2.0	17	8	5
IIIa	4.0	28	22	10
IVa	8.0	62	39	15

a. Serum with titers of less than 1:10 were assigned a value of 5.
b. The titer of this serum was 1:640.

When HAI titers had dropped to a value of <1:10, guinea pigs in both trials which had survived the schedule of repeated cardiac punctures were

challenged with approximately 10^3 guinea pig intraperitoneal median lethal doses ($GPIPLD_{50}$) of Trinidad strain virus. Of the 25 animals challenged, 9 succumbed. Of the 16 survivors, 13 developed HAI titers ranging from 1:320 to 1:5120, indicating that these animals had been infected. The HAI titers of 2 survivors remained unchanged following challenge while the remaining survivor was not bled.

From this study, it was evident that, even though HAI titers were <1:10 at time of challenge, sufficient passive antibody remained to protect against the lethal effects of the VEE virus infection.

In an effort to define more exactly the duration of protection afforded by passively administered antibody, 60 guinea pigs were each inoculated IP with 4.0 ml of homologous VEE immune serum. This serum had an HAI titer of 1:1280 and an SNI >7 logs. Twenty-four hr after administration of serum and at weekly intervals thereafter, 6 guinea pigs were selected from the group, bled for serology, and challenged with approximately 10^3 $GPIPLD_{50}$ of Trinidad strain virus. The results are presented in Table VI.

Seventy-one days following the administration of serum, 14 animals which had survived the original exposure to unmodified virus were re-challenged. In addition to surviving the initial challenge these animals had not shown an increase in antibody titer. Their HAI titers at the time of the second challenge were all <1:10. Of the 14, one animal from Group X survived. However, since only 8 days separated the 2 challenges, this animal may have been developing antibody as a result of the first challenge. This conclusion is supported by the fact that 2 other animals in this group showed evidence of responding within the 8-day period, one having a titer of 1:10 and the other a titer of 1:40.

In addition to HAI serology, serum neutralization tests were performed on several of the pre-challenge sera. With an occasional exception, only those animals having a SNI of 1.6 logs or greater survived the challenge dose of approximately 10^3 $GPIPLD_{50}$.

Under the conditions of the study described, the duration of protection afforded by the administration of passive antibody was between 63 and 70 days. Antibody titers of animals challenged within the first 2 weeks after administration of serum usually decreased in the same manner as those of animals receiving serum alone. This decrease is taken as evidence that infection was prevented, and that the quantity of antigen inoculated did not elicit an antibody response. In contrast, antibody titers of animals challenged 30 to 60 days after administration of serum usually rose to high levels, indicating that infection had occurred.

The next experiment was similar to that involving monkeys. At intervals of 0, 4, 8, 24, 48, and 72 hr after inoculation of 10^3 $GPIPLD_{50}$ of Trinidad strain virus, 6 guinea pigs were each administered 4.0 ml of homologous VEE immune serum via the IP route. The HAI titer of this serum

TABLE VI. DURATION OF PROTECTION AFFORDED BY PASSIVELY ADMINISTERED HOMOLOGOUS VEE IMMUNE SERUM IN THE GUINEA PIG

GROUP NO.	DAYS AFTER SERUM ADMINISTRATION	MEAN[a] RECIPROCAL HAI TITER	SURVIVORS TOTAL[b]	SURVIVORS SHOWING INCREASE IN TITER OVER SERUM CONTROL[b]
I	1	76	5/6	1/5
II	7	53	6/6	0/3
III	15	28	6/6	1/5
IV	22	13	4/6	2/4
V	29	8	6/6	5/5
VI	36	8	6/6	4/5
VII	42	5	4/6	4/4
VIII	50	5	4/6	4/4
IX	57	5	2/5	2/2
X	63	5	3/4	2/3

a. For purposes of computation, a value of 5 was assigned to sera with titers of less than 1:10 (the lowest dilution used in the HAI test).

b. The numbers of survivors in the last two columns do not agree in every case because some survivors died from bleeding before completion of the experiment.

was 1:1280 and the SNI >7.0 logs. Results are presented in Table VII. The numbers of survivors in the last two columns do not agree in every case because some survivors died from bleeding before completion of the scheduled bleedings.

From Table VII it can be seen that guinea pigs were protected against the lethal effects of virulent VEE virus infection when homologous VEE immune serum was administered within 24 hr after virus inoculation.

HAI tests performed on sera obtained in serial bleedings of these animals, subsequent to the administration of serum, showed that the rate and magnitude of antibody response varied as a function of the time between inoculation of virus and serum, respectively. These results are summarized in Figure 1. The antibody titers of animals administered serum immediately after virus inoculation decreased in the same manner as those of animals which received serum

TABLE VII. EFFECT OF PASSIVE ANTIBODY ON VEE INFECTION IN GUINEA PIGS

GROUP NO.	TIME SERUM ADMINISTERED AFTER TRINIDAD INOCULATION (hr)	NO. SURVIVED / TOTAL	SURVIVORS SHOWING INCREASE IN TITER OVER SERUM CONTROLS
I	0	6/6	0/4
II	4	5/6	3/5
III	8	5/6	2/4
IV	24	6/6	6/6
V	48	0/6	-
VI	72	0/6	-
VII	No serum	0/6	-

alone. In contrast, antibody titers of animals treated 24 hr after inoculation of virus rose rapidly to high levels. The animals administered serum at 4 and 8 hr after virus are also of interest. Approximately 50% of the animals in these groups responded with an increase in titer (similar to the group treated at 24 hr). However, the antibody titers of animals in the 4- and 8-hr groups which responded did not rise as rapidly or to as high levels as did those of the 24-hr group. It should be noted that the curve for "None - all groups" represents 8 nonresponding animals from the 0, 4, and 8-hr groups.

Summary and Conclusions:

The half-life of monkey and guinea pig HAI antibody in the homologous species was found to be in substantial agreement with published values for the half-lives of the respective globulins, i.e., 6.6 days for the monkey and 5.4 days for the guinea pig (J Exp Med 96:313, 1952).

From the results of the study in monkeys it would appear that infection was established as early as 4 hr after inoculation of virus. Administration of immune serum at this time served to delay the antibody response. At 24 hr after virus inoculation, administration of serum resulted in the clearing of over 4 logs of virus from the circulation.

When present in relatively large amounts, passive antibody blocked infection in guinea pigs, and titers decreased in the same manner as those of animals which received serum alone.

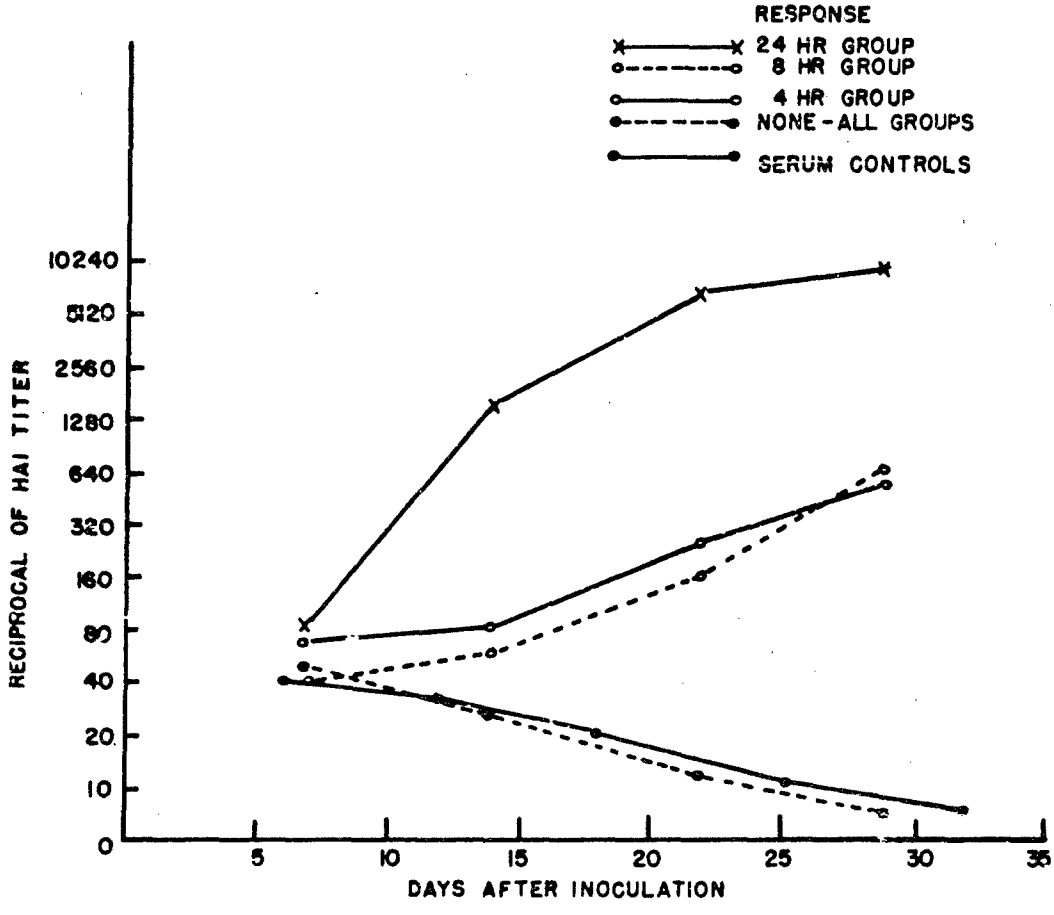

FIGURE 1. HAI TITERS IN ANIMALS ADMINISTERED SERUM AFTER INOCULATION OF VIRULENT VEE VIRUS.

If the passive antibody was allowed to decline, a level was reached at which infection did occur, but lethal consequences were prevented. Evidence of infection was provided by the rapid rise of antibody titers.

When passive antibody was present in lesser amounts, infection with VEE proceeded unaltered.

Administration of passive antibody was efficacious in the treatment of VEE infection up to 24 hr following inoculation of virus. The rate and magnitude of antibody response varied as a function of the time between inoculation of virus and serum, respectively.

Publications:

None.

ANNUAL PROGRESS REPORT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-01:	Vulnerability of Man to Biological Warfare
Study No. -40-08:	Host-parasite Relationships in Venezuelan Equine Encephalomyelitis
Reporting Installation:	U. S. Army Medical Unit Fort Detrick, Maryland
Division:	Animal Assessment and Virology
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Authors:	Arthur L. Hogge, Jr., Colonel, VC (Sect. I, II, III) Robert W. McKinney, Major, MSC (Sect. I, II, III, IV) William C. Day, Captain, VC (Sect. I) Melvin H. Davis, Lieutenant, VC (Sect. I) Ralph E. Thomas, Major, VC (Sect. II, III) Herbert L. Morton, Lieutenant, VC (Sect. II) Telford H. Work, MD (CDC) (Sect. IV) Roy W. Chamberlain, PhD (CDC) (Sect. IV) Edwin C. Corristan (USABL) (Sect IV)
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-01: Vulnerability of Man to Biological Warfare

Study No. -40-08: Host-parasite Relationships in Venezuelan Equine Encephalomyelitis

Section I: Evaluation of the Trinidad Strain of VEE in Swine

Reporting Installation: U. S. Army Medical Unit
Fort Detrick, Maryland

Division: Animal Assessment

Period Covered by Report: 1 July 1963 to 30 June 1964

Professional Authors: Arthur L. Hogge, Jr., Colonel, VC
Robert W. McKinney, Major, MSC
William C. Day, Captain, VC
Melvin H. Davis, Lieutenant, VC

Reports Control Symbol: RCS-MEDDH-288

Security Classification: UNCLASSIFIED

The challenge of young Duroc pigs with Trinidad Venezuelan equine encephalomyelitis (VEE) virus was undertaken to study concentration in the blood, liver, and brain. The greatest level of blood virus found was $10^{2.38}$ $MIPLD_{50}$/0.3 ml. This level of viremia is not high enough to suggest that swine are important as virus sources for mosquitos. The 10% suspensions of liver and brain tissue killed only a portion of mice injected indicating a low level of virus activity.

The experimental infection of pregnant swine did not cause abortions. It was also demonstrated that VEE virus can be isolated from the milk of lactating sows up to 5 days following challenge. The infection of swine with VEE resulted from the housing of susceptible pigs with experimentally infected pigs.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-01: Vulnerability of Man to Biological Warfare

Study No. -40-08: Host-parasite Relationships in Venezuelan Equine Encephalomyelitis

Section J: Evaluation of the Trinidad Strain of VEE in Swine

Description:

To investigate various interactions between an animal host and Venezuelan equine encephalomyelitis.

Progress:

In FY 1963 Annual Progress Report we reported the susceptibility of swine to Venezuelan equine encephalomyelitis (VEE) and the clinical responses illicited following experimental inoculation of VEE virus. The current studies were designed to extend the knowledge gained from that work.

Four classes of Duroc swine were employed in these studies. These were: young pigs 3-1/2 months of age, young adult swine 8 months of age, adult pregnant sows, and lactating sows that had recently farrowed.

The virus used for these studies was the Trinidad strain of VEE. Employing the method of Reed and Muench, doses of viruses employed were determined by titrating intraperitoneally (IP) 10-fold serial dilutions of the stock virus in 12-14-gm albino Swiss mice. The challenge of swine was by intramuscular (IM) injection. In all instances the diluent used for making dilutions was phosphate buffered saline (PBS) with 1% normal rabbit serum (NRS).

The demonstration of virus in specimens collected from these swine was accomplished by titrating materials IP in 12-14-gm albino Swiss mice.

Ten young pigs (3-1/2 months of age) were inoculated IM with 10^5 $MIPLD_{50}$ of virus in a serial sacrifice study. Two animals were sacrificed each day beginning day 1 (24 hr postchallenge) and extending through day 5.

The animals were bled daily for viremia studies. At time of sacrifice 10% suspensions of liver and brain were prepared from each pig and injected IP into mice.

During this study blood virus activity was demonstrated in all swine except one (No. 45). Only 2 of 60 mice injected IP with 0.3 ml of brain suspension died. Fourteen of 60 receiving 0.3 ml of liver suspensions died. This indicates that the level of virus in these tissues is not great.

The relationship between virus levels in the blood, brain, and liver of these animals is presented in Table I.

TABLE I. DISTRIBUTION OF VEE VIRUS IN SACRIFICE STUDY OF SWINE AS DETERMINED BY MOUSE INOCULATION (6 MICE/GROUP)

	NO. MICE DEAD AFTER IP INOCULATION OF TISSUES[a] ON DAY														
PIG	1			2			3			4			5		
NO.	Bl.	Br.	Li.	Bl.	Br.	Li.	Bl.	Br.	Li.	Bl.	Br.	Li.	Bl.	Br.	Li.
91	6	1	2												
59	6	0	5												
88	6			3	0	2									
45	0			0	0	0									
52	6			6			1	0	3						
37	3			6			0	0	2						
47	6			0			0			0	0	0			
92	0			0			0			1	0	0			
94	0			1			4			0			0	1	0
56	6			5			5			1			0	0	0

a/ Bl = blood, 10^{-1} dilution.
Br = brain
Li = liver

At the time these pigs were challenged, 2 swine, comparable in age and size, were not inoculated but placed in the pen with the challenged animals. These animals were bled daily for 6 days for viremia determinations, temperatures were taken twice daily, and sera tested for hemagglutination inhibiting (HAI) antibodies.

One of the 2 had a frank fever on day 4; both pigs manifested what was considered a low-grade fever from days 1-5 and 1-4. Prior to the study the HAI titer of both was found to be <1:10; when examined 24 days later one had an HAI titer of 1:2560 and the other 1:320.

A low level viremia developed in both pigs, demonstrated by injecting 0.3 ml whole blood IP into mice. Blood collected on days 4 and 6 from 1 pig killed 2/6 and 3/6 mice, respectively. Blood obtained from the other pig on day 6 killed 1/6 mice. Based on these observations, there is no doubt that these pigs became infected. However, the exact mode of transmission was not defined.

In another serial sacrifice study, 8 young adult swine (8 months of age) were inoculated IM with 10^6 $MIPLD_{50}$ of Trinidad VEE virus. Two animals were sacrificed each day beginning day 1 and extending through day 4. Whole blood and 10% suspensions of liver and brain tissue were titrated in mice. Rectal temperatures were taken twice daily. The results of this study are presented in Table II.

Eight adult pregnant sows were inoculated with VEE virus and observed for abortions. Three sows received $10^{5.7}$ $MIPLD_{50}$ of the Trinidad strain of VEE and farrowed shortly after challenge. One farrowed on day 6, 1 on day 12, and 1 on day 14. A total of 15 pigs were born. All were alive and normal in appearance.

Three sows in the first semester of pregnancy (30, 40, and 60 days) were challenged with Trinidad VEE virus, 2 received 10^6 $MIPLD_{50}$ and 1 received 10^9 $MIPLD_{50}$. These animals were sacrificed just prior to parturition, but it was evident that they did not abort following the virus challenge. The 2 remaining sows received $10^{5.7}$ $MIPLD_{50}$ of virus. One was sacrificed on day 2 and the other on day 3 postchallenge. At time of sacrifice the brains and placentas of 3 fetuses from each sow were titrated in mice. A 10% suspension was made from these tissues and injected IP into each of 6 mice. There was only 1 mouse death, and this resulted from the injection of placental suspension.

These data indicate that the virus does not cross the placental barrier and becomes established in the fetal brain. The results further suggest that abortions of swine due to this virus are not likely. It should be remembered however, that these studies did not encompass the effects of infection during all stages of pregnancy.

Two lactating sows were inoculated with $10^{5.7}$ $MIPLD_{50}$ of Trinidad VEE virus and their blood and milk checked daily for the presence of virus from day 1 through day 5 postchallenge. In only 1 of the sows could virus be demonstrated. It is interesting to note that virus in the milk from this sow was demonstrated 1 day longer than in the blood. Table III summarizes these virus determinations.

TABLE II. FEVER AND TRINIDAD VEE VIRUS DISTRIBUTION IN SWINE SACRIFICE STUDY.

	PEAK TEMPERATURE[a] AND VIRUS TITER IN TISSUES (Log_{10})[b]															
	Day 1				Day 2				Day 3				Day 4			
PIG NO	Peak temp °F	Bl	Br	Li	Peak temp °F	Bl	Br	Li	Peak temp °F	Bl	Br	Li	Peak temp °F	Bl	Br	Li
1A-4	4.4	1.5	<1.0	1.5												
1A-6	2.0	1.8	-	1.3												
1A-1	2.8	1.8			2.6	<1.0	-	-								
1A-2	3.2	<1.0			3.8	1.0	-	-								
1A-3	2.6	<1.0			4.0	<1.0			1.4	<1.0	<1.0	-				
1A-5	3.4	2.6			3.0	2.4			4.6	-	-	-				
1A-7	3.4	-			4.8	-			3.2	-			2.6	-	-	-
1A-8	4.2	1.0			5.4	<1.0			5.2	-			4.2	-	-	-

a/ 100°F +

b/ Bl = blood
Br = brain
Li = liver

TABLE III. RESPONSES OF LACTATING SOWS TO TRINIDAD STRAIN VEE VIRUS

INOCULATION DAY	S-14 Peak temp, °F	S-14 No. Mice Dead[a/] Blood	S-14 No. Mice Dead Milk	S-15 Peak temp, °F	S-15 No. Mice Dead Blood	S-15 No. Mice Dead Milk
1	103.4	6	6	103.2	3	0
2	102.4	6	1	102.0	0	0
3	104.2	6	5	104.8	0	0
4	104.0	0	5	105.8	0	0
5	102.8	0	2	106.6	0	0
6	103.2	0	0	102.4	0	0

a/ 0.3 ml whole blood or milk inoculated IP into 6 mice.

The sow that had demonstrable virus in her milk was nursing 5 pigs. These pigs were bled 14 days following the dam's challenge and all had positive HAI antibody titers ranging from 1:640 to 1:10,240. The other sow had 11 nursing pigs. When the pigs were checked 14 days following challenge of the sow, 7 of 11 had demonstrable HAI titers ranging from 1:640 to 1:5120. None of the pigs died or exhibited clinical signs of illness.

Summary and Conclusions:

The challenge of young Duroc pigs with Trinidad VEE virus was undertaken to study virus concentrations in the blood, liver, and brain.

The greatest level of blood virus found was $10^{2.38}$ $MIPLD_{50}$/0.3 ml. This level of viremia is not high enough to suggest that swine are important as virus sources for mosquitos. The 10% suspensions of liver and brain tissue killed only a portion of mice injected indicating a low level of virus activity.

The experimental infection of pregnant swine did not cause abortions. It was also demonstrated that VEE can be isolated from the milk of lactating sows up to 5 days following challenge. The infection of swine with VEE resulted from housing of susceptible pigs with experimentally infected pigs.

Publications:

None.

ABSTRACT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-01: Vulnerability of Man to Biological Warfare

Study No. -40-08: Host-parasite Relationships in Venezuelan Equine Encephalomyelitis

Section II. Attenuated VEE Virus in Pregnant Swine.

Reporting Installation: U. S. Army Medical Unit
Fort Detrick, Maryland

Division: Animal Assessment

Period Covered by Report: 1 July 1963 to 30 June 1964

Professional Authors: Arthur L. Hogge, Jr., Colonel, VC
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Reports Control Symbol: RCS-MEDDH-288

Security Classification: UNCLASSIFIED

The administration of attenuated VEE virus to pregnant swine did not cause abortions. At time of farrowing a large percentage of the young pigs were stillborn. After extensive study it was concluded that these sows had become infected with brucellosis which could account for the abnormal farrowings. The baby pigs born of these sows had negative (<1:10) hemagglutination-inhibiting antibody titers at birth but developed positive titers subsequent to nursing. These titers were again negative (<1:10) when the pigs were 2-1/2 months of age. When challenged at this time with Trinidad VEE virus the pigs were shown to be partially protected.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-01: Vulnerability of Man to Biological Warfare

Study No. -4C-08: Host-parasite Relationships in Venezuelan Equine Encephalomyslitis

Section II: Attenuated VEE Virus in Pregnant Swine.

Description:

To investigate various interactions between an animal host and Venezuelan equine encephalomyelitis.

Progress:

The infection of pregnant dams with certain viral agents has been observed to cause adverse effects on the developing fetuses. This phenomenon is seen when women become infected with rubella; sheep, with bluetongue, equines, with rhinopneumonitis; and swine, with hog cholera.

The development of an attenuated strain of Venezuelan equine encephalomyelitis (VEE) virus intended as a vaccine required that studies be conducted to determine if undesirable effects resulted from the use of this vaccine strain in pregnant animals. The selection of swine as the experimental model was based on the knowledge that teratogenic defects were commonly observed when pregnant sows were vaccinated with attenuated hog cholera virus.

Two strains of VEE virus were employed in these studies. The attenuated strain had a passage history of GPH78/CF2/GPH13. The unmodified Trinidad strain used was 13th passage chick embryo slurry.

The viruses were titrated in 12-14-gm albino Swiss mice by the intraperitoneal (IP) route. The doses of viruses employed were determined by the method of Reed and Muench. Mice were also used for viremia determinations. Hemagglutination-inhibiting (HAI) antibody responses were determined by the method of Clarke and Casals.

The swine were young adult Durocs that had not previously been bred. The drove consisted of 1 boar and 13 females. The sows were bred and dates of breeding recorded. Nine of the sows were subsequently administered attenuated VEE virus intramuscularly (IM) and 4 served as uninoculated controls. Seven of the 9 inoculated sows received $10^{3.87}$ $MIPLD_{50}$ of virus. At time of vaccination these sows were from 22-32 days pregnant. The remaining 2 were administered $10^{5.86}$ $MIPLD_{50}$ of virus when they were 48 and 49 days pregnant.

Preinoculation sera were all negative for HAI antibody. There were no clinical manifestations of illness observed following administration of the attenuated virus. The animals remained afebrile and did not abort. The sows became infected based on the development of positive HAI titers subsequent to vaccination. Sera were obtained several times during the postchallenge inoculation period and each specimen was found positive for HAI antibodies.

The serologic responses of these swine are summarized in Table I. It is interesting to note that the level of HAI antibody did not change significantly during the time frame involved.

TABLE I. SEROLOGIC RESPONSES OF SWINE INOCULATED WITH ATTENUATED VEE VIRUS

DOSE OF ATTENUATED VIRUS Log_{10}	SOW NO.	RECIPROCAL HAI ANTIBODY TITER Day postinoculation 21-22	56-58	80-86
3.87	45	640	320	640
	37	40	40	40
	36	160	40	
	21	160	160	
	26	160	80	
	34	160	80	
5.86	25	160		
	10	640		
	14	320		

These 9 sows farrowed following a normal gestation period. At time of farrowing the baby pigs were examined for defects, and prior to nursing bled for HAI antibody determinations. All pigs tested prior to nursing had HAI titers of <1:10, but after nursing, developed positive titers.

When the sows farrowed a high per cent of stillbirths were observed. Fortunately, 1 of the 4 uninoculated sows farrowed at the same time. Her parturition was complicated and required assistance. Four dead pigs were delivered, 2 of which were underdeveloped. It was suspected that the swine had become infected with leptospirosis, burcellosis, or some other pathogen.

Specimens of serum and urine from the sows and the boar were tested for leptospirosis and found negative. When sera were tested for brucella agglutinins the boar was found to have a titer of 1:400 and the sows had titers ranging up to 1:200.

It was assumed, therefore, that these swine were infected with brucella and that the sacrifice of the adult animals was indicated because of public health implications. Attempts to isolate brucella from these animals were unsuccessful. The sacrifice of these animals was accomplished prior to the farrowing of the 3 remaining control sows. Necropsy of these control sows revealed evidence of pathologic changes compatible with brucella infections: there were abscess formations and 1 sow's uterus showed evidence that her pregnancy had terminated by a recent abortion or that the fetuses had been readsorbed. It is also of interest to note that these 3 control sows were bred several times and should have conceived and farrowed several weeks prior to sacrifice. This was also strong evidence supporting the theory of a brucella infection.

Twenty-nine of the 67 pigs farrowed by the vaccinated sows were stillborn. At time of birth all pigs were bled from the heart, some were sacrificed for pathology studies, some were denied the dam's milk and fed by bottle and some were left with the sows. Thirty-eight sera from these baby pigs were tested for HAI antibody determinations at birth and all were negative (<1:10). Farrowing data of vaccinated sows are presented in Table II.

TABLE II. FARROWING DATA OF PREGNANT SOWS ADMINISTERED ATTENUATED VEE VIRUS.

SWINE NO.	INOCULATION ATTENUATED VIRUS (day of pregnancy)	NO. PIGS BORN DEAD	NO. PIGS BORN ALIVE
37	29	0	6
45	32	2	1
36	27	5	8
25	26	5	8
34	26	2	10
26	25	11	1
21	22	1	0
10	48	1	2
14	49	2	2

Subsequent to nursing (2-3 weeks of age) the sera of 6 pigs that were permitted to nurse were tested for HAI antibodies and all were positive. The titers ranged from 1:40 to 1:320. Their sera were again tested when the pigs were approximately 2-1/2 months of age and the titers were all <1:10.

At this time the pigs were challenged with $10^{5.6}$ $MIPLD_{50}$ of unmodified Trinidad VEE virus. Six additional pigs, comparable in age and size were inoculated with the same dose and served as controls. Rectal temperatures were recorded twice daily for 10 days, viremia determinations were made day 1-4. Viremia was demonstrated in all control pigs, whereas virus was

recovered from only 1 of the pigs born of the vaccinated sows; the level of viremia was very low and demonstrable only on days 1 and 2 postchallenge. On each of these days 0.3 ml of whole blood killed only 1 of 6 mice infected IP. A febrile response was observed in all pigs.

The sera collected from these pigs were tested for HAI antibodies 21 days postchallenge, and all were positive. The titers ranged from 1:160 to 1:1280 in the principals while all controls had a titer of 1:1280. The responses of these pigs are summarized in Table III.

These data indicate that the young pigs born of vaccinated sows were resistant to challenge with unmodified Trinidad VEE vrius. The resistance observed was probably due to passive immunity obtained from the dam through the colostrum. It is interesting to note, however, that the sera tested prior to challenge did not have demonstrable HAI antibodies at the 1:10 dilution.

Summary:

Pregnant Duroc swine were administered attenuated VEE virus to determine if teratogenic defects and/or abortions would occur. No abortions were observed but when the sows farrowed a large percent of stillbirths were observed. This also occurred in 1 of the uninoculated controls. The cause of these stillbirths was believed to be due to brucellosis. This assumption was based principally on the demonstration of positive brucella titers. The animals were sacrificed and attempts to isolate brucella were unsuccessful. It was found that the HAI antibody titers of these sows did not change significantly from 21-80 days postinoculation. Sera from the young pigs born of these sows were tested for HAI antibodies at birth, and prior to nursing, they were all negative (<1:10) but were positive following nursing. At 2-1/2 months with unmodified Trinidad VEE virus, they were partially protected. They had a febrile response, but from only 1 could virus be isolated from blood.

Publications:

None.

TABLE III. RESPONSES OF YOUNG SWINE TO TRINIDAD VEE (105.6 $MIPLD_{50}$, IM)

PIG NO.	PEAK TEMPERATURE, 103°F +							VIREMIA No. of Mice Dead of 6 on days[b]				RECIPROCAL HAI TITER			
	0[a]	1	2	3	4	5	6	1	2	3	4	At birth	3 wks of age	Pre[c] chall	Day 24
YOUNG SWINE FROM VACCINATED SOWS															
34-1	2.2	3.4	3.0	2.0	3.0	2.2	3.0	0	0	0	0	-	160	-	160
34-2	3.4	3.0	3.6	2.0	3.0	4.0	3.2	0	0	0	0	-	80	-	160
34-3	3.0	4.6	4.8	5.0	4.4	4.4	3.2	1	1	0	0	-	40	-	1280
34-4	4.6	3.6	2.4	4.4	3.8	3.0	2.6	0	0	0	0	-	80	-	640
36-1	6.6	4.6	3.8	4.0	2.6	3.4	2.0	0	0	0	0	ND[d]	40	-	640
45-2	3.0	3.4	3.2	2.8	3.4	2.6	2.6	0	0	0	0	-	80	-	640
CONTROLS															Day 21
D-38	1.0	3.2	3.2	3.8	3.2	2.6	2.4	6	6	2	0			-	1280
D-39	2.0	4.4	2.4	3.6	3.6	1.4	1.4	5	6	0	0			-	1280
D-40	3.0	5.0	2.6	3.6	2.4	4.2	3.6	5	0	1	0			-	1280
D-53	4.0	5.0	4.6	3.8	3.8	3.6	2.8	5	4	0	0			-	1280
D-54	1.6	3.8	4.6	4.8	2.8	2.0	2.6	6	6	1	2			-	1280
D-55	2.6	3.2	2.8	5.4	4.4	2.6	2.8	6	2	0	0			-	1280

[a] 8-hr postchallenge.
[b] 0.3 ml whole blood, IP.
[c] Age 2-1/2 months.
[d] Not done.

ABSTRACT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-01: Vulnerability of Man to Biological Warfare

Study No. -40-08: Host-parasite Relationships in Venezuelan Equine Encephalomyelitis

Section III: Attenuated VEE Virus in Pregnant Guinea Pigs

Reporting Installation: U. S. Army Medical Unit
Fort Detrick, Maryland

Division: Animal Assessment

Period Covered by Report: 1 July 1963 to 30 June 1964

Professional Authors: Arthur L. Hogge, Jr., Colonel, VC
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Reports Control Symbol: RCS-MEDDH-288

Security Classification: UNCLASSIFIED

This study indicates that administration of the attenuated strain of Venezuelan equine encephalomyelitis (VEE) virus to pregnant guinea pigs does not cause undesirable effects. The pigs born of these sows were normal in appearance and the per cent stillbirths observed do not exceed that normally seen in a breeding colony. The number of pigs derived from the sows is also compatible with that observed in a breeding colony.

The resistance of the young pigs to unmodified Trinidad VEE virus is clear evidence that immunity had been acquired. This immunity may have been attained by maternal transfer of antibodies; at time of challenge sufficient levels were present to protect the animal.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-01: Vulnerability of Man to BW

Study No. -40-08: Host-parasite Relationships in Venezuelan Equine Encephalomyelitis

Section III: Attenuated VEE Virus in Pregnant Guinea Pigs.

Description:

To investigate various interactions between an animal host and Venezuelan equine encephalomyelitis.

Progress:

An isolated colony of Hartley strain guinea pigs was established to determine if the administration of an attenuated strain of Venezuelan equine encephalomyelitis (VEE) virus to pregnant sows would cause undesirable effects.

Two strains of VEE were used in these studies: the attenuated strain was produced by the Virology Division, USAMU, and identified as TC-83/3-2. The second strain of virus used was the unmodified Trinidad strain.

Titer of the attenuated virus was established as follows: Ten-fold serial dilutions of the attenuated virus were made and 0.3 ml of each dilution was injected intraperitoneally (IP) into each of 6 mice. Two weeks later these mice were inoculated IP with $10^{2.3}$ $MIPLD_{50}$ of the Trinidad strain of VEE. The Trinidad strain of virus was also titrated in mice. Doses of virus were calculated using the method of Reed and Muench.

The guinea pigs were bred and dates of breeding recorded. A total of 26 females were successfully bred during the study. These sows were inoculated IP with $10^{4.88}$ $MIPLD_{50}$ of the attenuated virus. At time of challenge, the stages of pregnancy ranged from 12-36 days.

Of the 26 pigs challenged only 1 aborted during the experiment. This occurred 14 days postinoculation and the necropsy report indicated that death was due to pneumonia.

The 25 sows gave birth to a total of 88 pigs. The size of litters ranged from 1-5 pigs with a mean of 3.4. There were 4 stillborn pigs and 4 pigs died within 5 days of birth. One died at the age of 1 month. The animals dying were necropsied by Pathology Division, USAMU, and no evidence of CNS involvement was found. These deaths were attributed to pneumonia.

When these 78 young guinea pigs were weaned they were challenged with 10^3 $MIPLD_{50}$ of unmodified Trinidad VEE virus. At time of challenge the ages of these animals ranged from 9-12 weeks. Ten pigs, comparable in size and age were obtained and added to the group to serve as controls. Subsequent to the Trinidad challenge all controls died. Only 2 of the principals died. Both of these were late deaths, occurring on days 10 and 11 postchallenge. Attempts to demonstrate virus activity from the brains of the animals were unsuccessful. It was noted that approximately 10% of the survivors exhibited some evidence of illness during the 2-week observation period.

The young pigs were bled 14 days following challenge and sera checked for hemagglutination-inhibiting (HAI) antibodies. Approximately 80% had positive HAI antibody titers, some of which were found to be as high as 1:5120.

Summary:

The results of this study indicate that administration of the attenuated strain of VEE virus to pregnant guinea pigs does not cause undesirable effects. The pigs born of these sows were normal in appearance and the per cent stillbirths observed do not exceed that normally seen in a breeding colony. The number of pigs derived from the sows is also compatible with that observed in a breeding colony.

The resistance of the young pigs to unmodified Trinidad VEE virus is clear evidence that immunity had been acquired. This immunity may have been attained by maternal transfer of antibodies and at time of challenge sufficient levels were present to protect the animal.

Publications:

None.

ABSTRACT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-01:	Vulnerability of Man to Biological Warfare
Study No. -40-08:	Host-Parasite Relationships in Venezuelan Equine Encephalomyelitis Section IV. Arthropod transmission.
Reporting Installation:	U. S. Army Medical Unit Fort Detrick, Maryland
Division:	Virology
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Authors:	Robert W. McKinney, Major, MSC Telford H. Work, M.D., (CDC) Roy W. Chamberlain, Ph.D., (CDC) Edwin C. Corristan (USABL)
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

Twelve volunteers were inoculated with attenuated Venezuelan equine encephalomyelitis virus (VEE) vaccine. Beginning at 2 hr postinoculation and daily for the subsequent 14 days clinical observations were made and specimens for viral studies collected. During the same interval 35-40 Aedes triseriatus mosquitoes were fed daily on each subject. The mosquitoes were incubated for 18-20 days at which time they were allowed to feed on guinea pigs. None of the guinea pigs became infected as evidenced by serologic and challenge tests. Virus was not recovered with trituration of mosquitoes and inoculation into susceptible hosts.

From the results it appears unlikely that A. triseriatus or other mosquito of similar VEE infection threshold would become infected by feeding on persons with viremia following inoculation of attenuated VEE virus.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-01: Vulnerability of Man to Biological Warfare

Study No. -40-08: Host-Parasite Relationships in Venezuelan Equine Encephalomyelitis

Section IV. Arthropod transmission.

Description:

To investigate various interactions between animal hosts and the virus of Venezuelan equine encephalomyelitis.

Progress:

The cooperation and assistance of personnel of the Arbovirus Unit, Communicable Disease Center, Atlanta, Georgia and those of the Entomology Division, U.S. Army Biological Laboratories, Fort Detrick, Maryland is gratefully acknowledged. Particular thanks are extended to those persons who served as volunteers.

As reported last year successful infection of Aedes triseriatus and Aedes aegypti with an attenuated strain of Venezuelan equine encephalomyelitis (VEE) virus necessitated the use of relatively large quantities of virus.

Although attempts to infect these mosquitoes on viremic guinea pigs were negative, definitive studies in man were still required. An opportunity to perform these studies was afforded in relation to the immunization of personnel of the Arbovirus Unit at CDC.

Twelve persons scheduled for immunization volunteered for these studies. Table I lists their prior immunizations to other members of the group A arboviruses. Before inoculation of the attenuated virus, oral temperature, blood for serum and a throat swabbing were obtained from each person. These procedures were repeated at 2 hr and then daily for 14 days following inoculation. Each person was administered 0.5 ml of virus suspension subcutaneously which contained approximately 10^3 median guinea pig intraperitoneal immunizing doses ($GPIPID_{50}$).

TABLE I. IMMUNIZATION EXPERIENCE OF VOLUNTEERS ADMINISTERED ATTENUATED VEE VIRUS VACCINE

VOLUNTEER	VACCINE		
	EEE[a/]	WEE[b/]	VEE[c/]
Pt	+	+	-
Cr	-	-	-
We	+	+	-
So	-	-	-
La	+	+	-
En	+	+	+
Tl	+	+	-
Cn	+	+	+ [d/]
Wk	+	+	-
Li	+	+	-
Pn	+	+	-
Ce	+	+	-

a/ EEE - Eastern equine encephalitis, inactivated.
b/ WEE - Western equine encephalitis, inactivated.
c/ VEE - Live attenuated in 1961.
d/ VEE - Live attenuated in 1961 - no response.

Beginning at 2 hr and daily for 13 days after inoculation of virus, 35-40 A. triseriatus were allowed to feed on each subject. Feedings were carried out immediately following collection of the daily blood sample. The mosquitoes were then incubated at 80 F and 75% humidity for 18-20 days. During this period the daily blood samples as undiluted and a 1:10 dilution of serum were inoculated into suckling mice. The results of these assays were utilized as the basis for selecting the groups of mosquitoes for subsequent studies which were most likely to be infected.

The susceptibility of several laboratory hosts to infection with the attenuated virus is demonstrated by the results presented in Table II. These were derived from experiments designed to establish the validity of

viremia assay results and mosquito feedings.

TABLE II. SUSCEPTIBILITY OF SELECTED LABORATORY HOSTS TO INFECTION WITH ATTENUATED VEE VIRUS

TEST ANIMAL	ROUTE OF INOC.[a]	VOL. OF INOCULUM (ml)	LD_{50} TITER Per inoc. vol.	LD_{50} TITER Per cc.	ID_{50} AS REVEALED BY 21-DAY CHALLENGE[b] Per inoc. vol.	ID_{50} AS REVEALED BY 21-DAY CHALLENGE[b] Per cc.
Suckling mouse	IC	0.02	$10^{5.3}$	$10^{7.0}$		
Suckling mouse	SC	0.02	$10^{4.5}$	$10^{6.2}$		
3-week mouse	IC	0.03	$<10^{0.6}$[c]	--		
1/2-day chick	SC	0.03	$<10^{0.6}$	--		
G. Pig (400 gm)	IP	0.5	No deaths		$10^{6.1}$	$10^{6.4}$

a/ Route of inoculation - IC, intracerebral; SC, subcutaneous; IP, intraperitoneal.
b/ Challenge dose - $10^{3.2}$ adult mouse IC LD_{50} of Trinidad strain VEE virus via the IP route.
c/ One apparent breakthrough occurred in the 10^{-3} dilution.

Mosquitoes which had fed during period of apparent viremia were permitted to feed on 400-gm guinea pigs and were then frozen for subsequent testing for presence of virus. The guinea pigs were held for 30 days at which time they were bled for serum and then challenged with unmodified virus. The serum was tested for hemagglutination-inhibiting (HI) antibody. The challenge dose was $10^{3.2}$ mouse ICLD_{50} of Trinidad strain VEE virus administered by the intraperitoneal route.

The results of the viremia assay and mosquito experiments are presented in Table III. Despite the fact that mosquitoes were fed on the subjects at times of viremia, none of 344 transmitted virus to guinea pigs nor was virus demonstrated in 611 when triturated and inoculated into suckling mice.

Summary and Conclusions:

Under the conditions of this limited study no evidence was obtained that the infection in man with attenuated VEE virus is of sufficient magnitude to

provide for infecting mosquitoes, i.e., A. triseriatus.

While indicative that transmission of the attenuated VEE virus by mosquitoes from vaccinees is not likely to occur; experimental conditions in this study did not allow for mosquito assays at intervals between 0 and 18 days incubation.

This study will continue providing personnel and adequate facilities become available.

Publications:

None.

TABLE III. ATTEMPTED INFECTION OF A. TRISERIATUS BY FEEDING ON HUMAN SUBJECTS FOLLOWING INOCULATION WITH AN ATTENUATED STRAIN OF VEE VIRUS

SUBJECT	DAY OF FEEDING POST-INOCULATION	VIREMIA ASSAY[a] SERUM DILUTION None	1:10	MOSQUITOES Days extrinsic incubation	Number feeding on guinea pigs[b]	No. uninfected No. tested
Pt	2	6/8	?	18	16	0/29
	3	7/7	0/8	18	11[c]	0/27
	4	8/8	5/8	18	14	0/29
	5	6/3	1/8	18	14	0/2[illegible]
	7	0/8	8/8?	19	13	0/3[illegible]
Cr	2	0/8	2/8?	18	23	0/30
	3	0/8	1/8?	18	24	0/31
	5	0/7	1/8	18	18	0/32
We	2	2/2	0/7	18	16	0/26
	4	0/7	1/8?	18	11	0/27
	5	8/8	0/8	18	11	0/31
	9	0/3	1/8	19	13	0/25
	10	0/6	0/3	19	26	0/33
So	3	0/7	2/8?	18	15	0/22
	11	8/8	0/8	18	27	0/33
La	3	2/8?	8/8?	18	7	0/19
	4	1/6?	0/8?	18	13	0/31
	5	7/7	0/5	18	15	0/30
	6	8/8	1/8	20	19	0/27
	7	7/7	8/8	19	17	0/31
	8	2/8	1/8	19	15	0/28
	9	0/8	8/8	19	13	0/26
	11	0/7	1/7	18	17	0/27
En	3	3/8	0/8	18	11	0/30
Tl	3	3/8	3/8	18	8	0/14
	4	0/8	2/8?	18	12	0/21
	8	1/8	1/8	19	26	0/30
Cn	4	2/8	0/8	18	25	0/32
	5	5/8	0/8	18	7	0/32
	7	0/8	8/8?	19	21	0/35
	8	3/8	2/8	19	16	0/33

TABLE III. CONTINUED

SUBJECT	DAY OF FEEDING POST-INOCULATION	VIREMIA ASSAY[a] SERUM DILUTION None	1:10	MOSQUITOES Days extrinsic incubation	Number feeding on guinea pigs[b]	No. uninfected No. tested
Wk	4	2/10?	2/9?	18	19	0/29
	6	8/8?	0/8	20	20	0/28
Li	5	4/8	0/8	18	17	0/33
	7	0/8	2/8	19	17	0/35
Pn	5	5/8	0/8	18	13	0/29
	7	1/7	2/8	19	15	0/33
Cy	8	0/7	?	19	15	0/31
	9	4/8	0/7	19	15	0/27
	11	0/7	8/8?	18	21	0/29
	12	0/10	1/11	17	21	0/29

Total mosq. refed on guinea pigs with neg. results-----344

Total mosquitoes screened for virus with negative results----------611

a. No. suckling mice dying/no. suckling mice inoculated with either undiluted serum or serum diluted 1:10, drawn from the human subject immediately preceding the mosquito feeding. These figures may include nonspecific deaths, particularly where question-marked.

b. Absence of HI antibodies at 30 days of incubation, and lack of resistance to challenge against $10^{3.2}$ adult mouse $ICLD_{50}$ administered via the IP route was considered to indicate lack of VEE transmission by mosquito bite.

c. This guinea pig survived challenge but possessed no HI antibody. Since no virus could be detected in the mosquitoe which had fed upon this guinea pig, the survival of challenge was considered to be a freak occurrence.

ANNUAL PROGRESS REPORT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-02:	Prevention and Treatment of Biological Warfare Casualties
Study No. -00-01:	Chemotherapeutic Modification of the Immune Response
Reporting Installation:	U.S. Army Medical Unit Fort Detrick, Maryland
Division:	Medical
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Author:	William D. Sawyer, Major, MC
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project No. 1C622401A096:		Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A[illegible]-02:		Prevention and Treatment of Biological Warfare Casualties
Study No. -00-01:		Chemotherapeutic Modification of the Immune Response
Reporting Installation	:	U.S. Army Medical Unit Fort Detrick, Maryland
Division:	:	Medical
Period Covered by Report	:	1 July 1963 to 30 June 1964
Professional Author	:	William D. Sawyer, Major, MC
Reports Control Symbol	:	RCS-MEDDH-288
Security Classification	:	UNCLASSIFIED

No additional studies have been performed and none are contemplated.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-02: Prevention and Treatment of Biological Warfare Casualties

Study No. -00-01: Chemotherapeutic Modification of the Immune Response

Description:

Compounds known to inhibit antibody respon[illegible], such as purine and analoques (6 mercaptopurine or related compounds), will be utilized to assess the nature and significance of antibody production consequent to administration of microbial and related antigens in experimental animas and man.

Progress:

No additional studies have been performed and none are contemplated.

Summary:

No additional studies have been performed and none are contemplated.

Publications:

None

ANNUAL PROGRESS REPORT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-02:	Prevention and Treatment of BW Casualties
Study No. -00-02:	Evaluation of Efficacy of Experimental Vaccines.
Reporting Installation:	U. S. Army Medical Unit Fort Detrick, Maryland
Divisions:	Animal Assessment and Medical
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Authors:	Ralph W. Kuehne (Sect. I) William C. Day, Captain, VC (Sect. I) Arthur L. Hogge, Jr., Colonel, VC (Sect. I) William D. Sawyer, Major, MC (Sect. I, II) Thomas J. Smith, Major, MC (Sect. II) Robert W. McKinney, Major, MSC (Sect. II)
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-02: Prevention and Treatment of BW Casualties

Study No. -00-02: Evaluation of Efficacy of Experimental Vaccines.

Section I: The Efficacy of Viable Pasteurella tularensis Vaccine against Respiratory Challenge with Strains SCHU-S4 and SCHU-S5 in Macaca Mulatta.

Reporting Installation: U. S. Army Medical Unit
Fort Detrick, Maryland

Division: Animal Assessment

Period Covered by Report: 1 July 1963 to 30 June 1964

Professional Authors: Ralph W. Kuehne
William C. Day, Captain, VC
Arthur L. Hogge, Jr., Colonel, VC
William D. Sawyer, Major, MC

Reports Control Symbol: RCS-MEDDH-288

Security Classification: UNCLASSIFIED

Studies were conducted to evaluate the efficacy of viable attenuated Pasteurella tularensis vaccine (National Drug Co. Lot 6) in protecting monkeys against respiratory challenges of SCHU-S4 and SCHU-S5 strains of P. tularensis.

These studies do not establish whether or not there is a difference in protection afforded against strains SCHU-S4 and SCHU-S5, but do indicate a need for extending the observations to larger numbers.

BODY OF REPORT

Project No. 1C622401A09..: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-02: Prevention and Treatment of BW Casualties

Study No. -00-02: Evaluation of Efficacy of Experimental Vaccines.

Section I: The Efficacy of Viable Pasteurella tularensis Vaccine against Respiratory Challenge with Strains SCHU-S4 and SCHU-S5 in Macaca Mulatta.

Description:

To evaluate experimental vaccines developed by various organizations, contractors, or other governmental agencies.

Progress:

A search of the literature revealed surprisingly little information concerning the effectiveness of tularemia living vaccine (LVS) against the streptomycin-resistant strain of Pasteurella tularensis (SCHU-S5) and inconsistencies were noted in studies with the streptomycin-susceptible (SCHU-S4) strain. Table I summarizes these reports.

TABLE I. THE EFFICACY OF VIABLE P.TULARENSIS VACCINE (LVS) AGAINST RESPIRATORY CHALLENGE WITH STRAINS SCHU-S4 AND SCHU-S5 IN M. MULATTA.

LABORATORY	AEROSOL CHALLENGE DOSE	CONTROLS (%Mortality)	VACCINATES (%Infected)	VACCINATES (%Mortality)
SCHU-S5				
USABL[a] (1961)	750	87	100	40
SCHU-S4				
USAMU[b] (1958)	60	18	50	0
USABL (1962)	5	50	100	0
USAMU (1958)	300	55	50	13
USAMU (1958)	1,300	100	100	100
USABL (1959)	1,000-50,000	100	100	47
USABL (1962)	10,000-50,000	100	100	38
USAMU (1958)	100,000	100	100	55
USAMU (1958)	130,000	100	100	100

a/ U.S. Army Biological Laboratories, Fort Detrick, Md.
b/ U.S. Army Medical Unit

To reinforce the SCHU-S5 data, 10 Macaca mulatta were vaccinated intradermally with LVS (Lot ND-6) and challenged 130 days later by the respiratory route. At time of challenge, 7 unvaccinated monkeys were added to the study as controls. The exposures were made on the modified Henderson apparatus employing 2 dose levels: 2,000 and 22,000 cells. Five vaccinates were challenged at each dose level, with 4 controls exposed to the low dose and 3 to the high dose.

Following exposure the animals were observed for signs of illness and death. On day 24 postchallenge all survivors were sacrificed. All vaccinates exposed to the 2,000-cell dose exhibited signs of illness with deaths occurring in 2 (40%). All of the vaccinates exposed to the 22,000-dose level became ill and died. All 7 controls employed in this study became ill and died.

It was felt important to determine whether there was any significance to the fact that with a SCHU-S5 dose of 22,000, there were no survivors, yet previous studies with SCHU-S4 (Table I) showed a 62% survival in the 10,000 to 50,000 dose range, and 45% survival at 100,000.

A study was therefore undertaken to assess protection afforded by LVS vaccine against a challenge of SCHU-S4 compared with a challenge of SCHU-S5, employing the same vaccine, the same exposure apparatus, laboratory techniques, and animal handling and personnel.

Thirty-two rhesus monkeys were vaccinated intradermally with ND Lot No. 6 LVS vaccine and were bled for serology on day 56 postvaccination. On day 57, 16 vaccinated and 10 control animals were exposed to a dynamic aerosol of SCHU-S4 strain of P tularensis on the modified Henderson apparatus. Eight principals and 5 controls received an average dose of 240 cells; the rest received 6000 cells. On day 59, the other 16 vaccinates and 10 controls were exposed to an aerosol challenge of SCHU-S5 strain. Half received an average of 220 organisms, the remainder, 4500 cells. Temperatures were taken twice daily through day 28 postchallenge; the animals were x-rayed, and bled for CRP and blood cultures twice weekly during this period. Survivors were bled for serology on day 14 and at time of sacrifice on day 28. Febrile responses, CRP, X-ray findings, and mortalities are shown in Figures 1 through 4.

All vaccinated animals challenged at the 2 low dose levels survived. No deaths occurred in vaccinates receiving the high dose (6,000 cells) of SCHU-S4. However, of particular interest is the fact that 25% mortality was observed in the vaccinates receiving the high dose (4500 cells) of SCHU-S5. The 2 vaccinates which succumbed to the SCHU-S5 challenge showed positive agglutination titers prior to exposure.

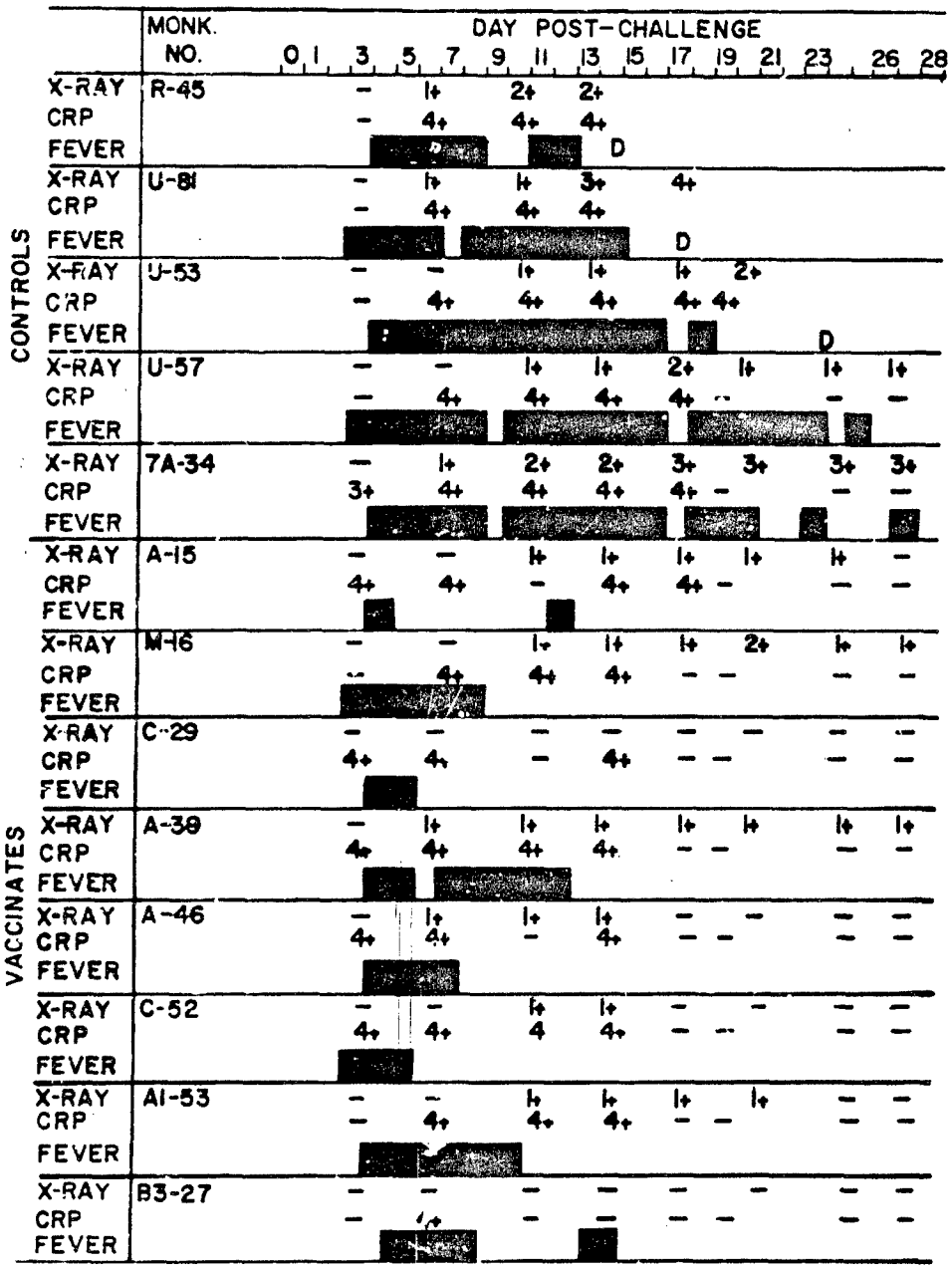

FIGURE 1. RESPONSES OF *M. MULATTA* TO AEROSOLIZED *P. TULARENSIS* SCHU-S4, LOW DOSE (240 CELLS).

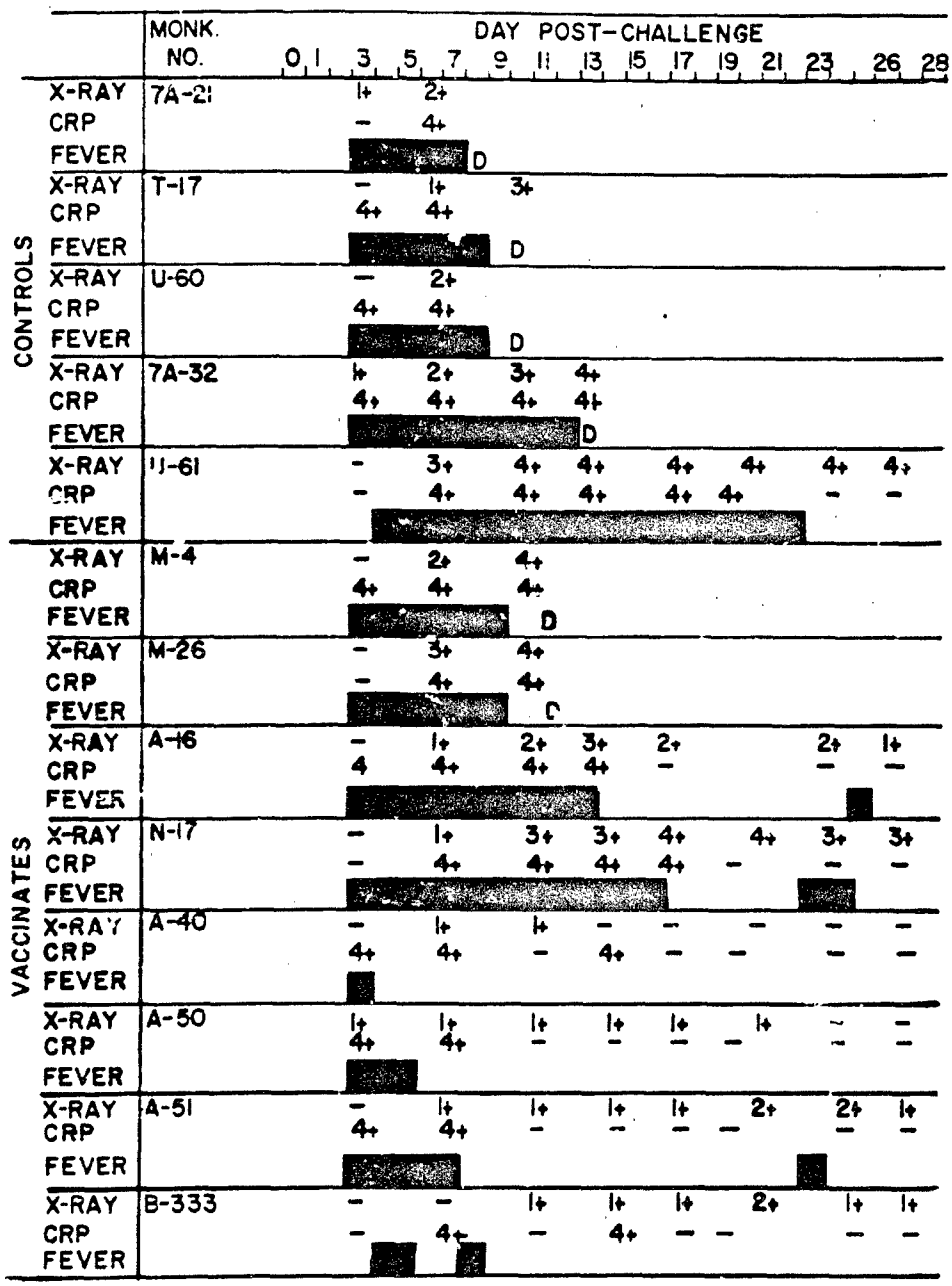

FIGURE 2. RESPONSES OF *M. MULATTA* TO AEROSOLIZED *P. TULARENSIS* SCHU-S4, HIGH DOSE (6000 CELLS).

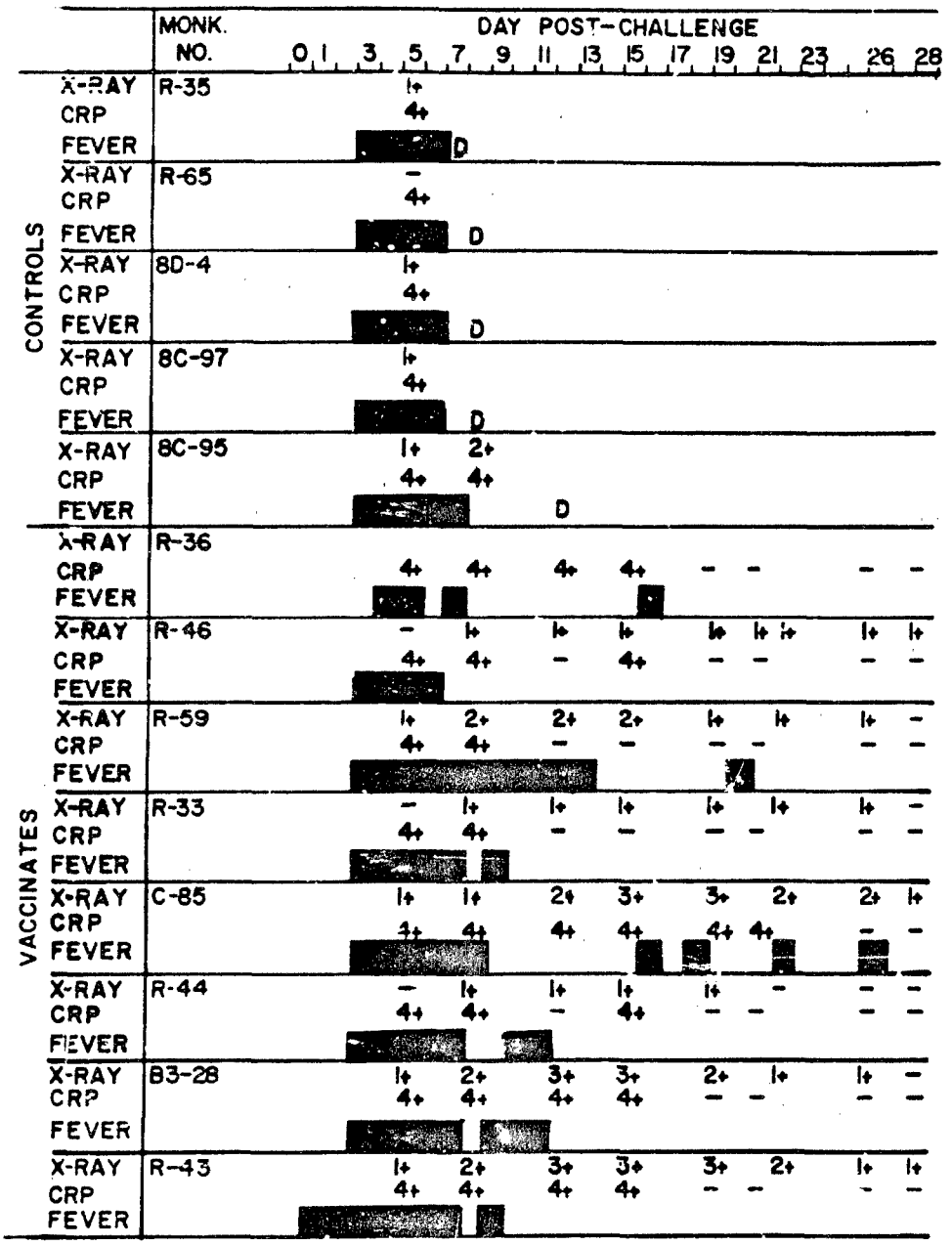

FIGURE 3. RESPONSES OF *M. MULATTA* TO AEROSOLIZED *P. TULARENSIS* SCHU—S5, LOW DOSE (220 CELLS).

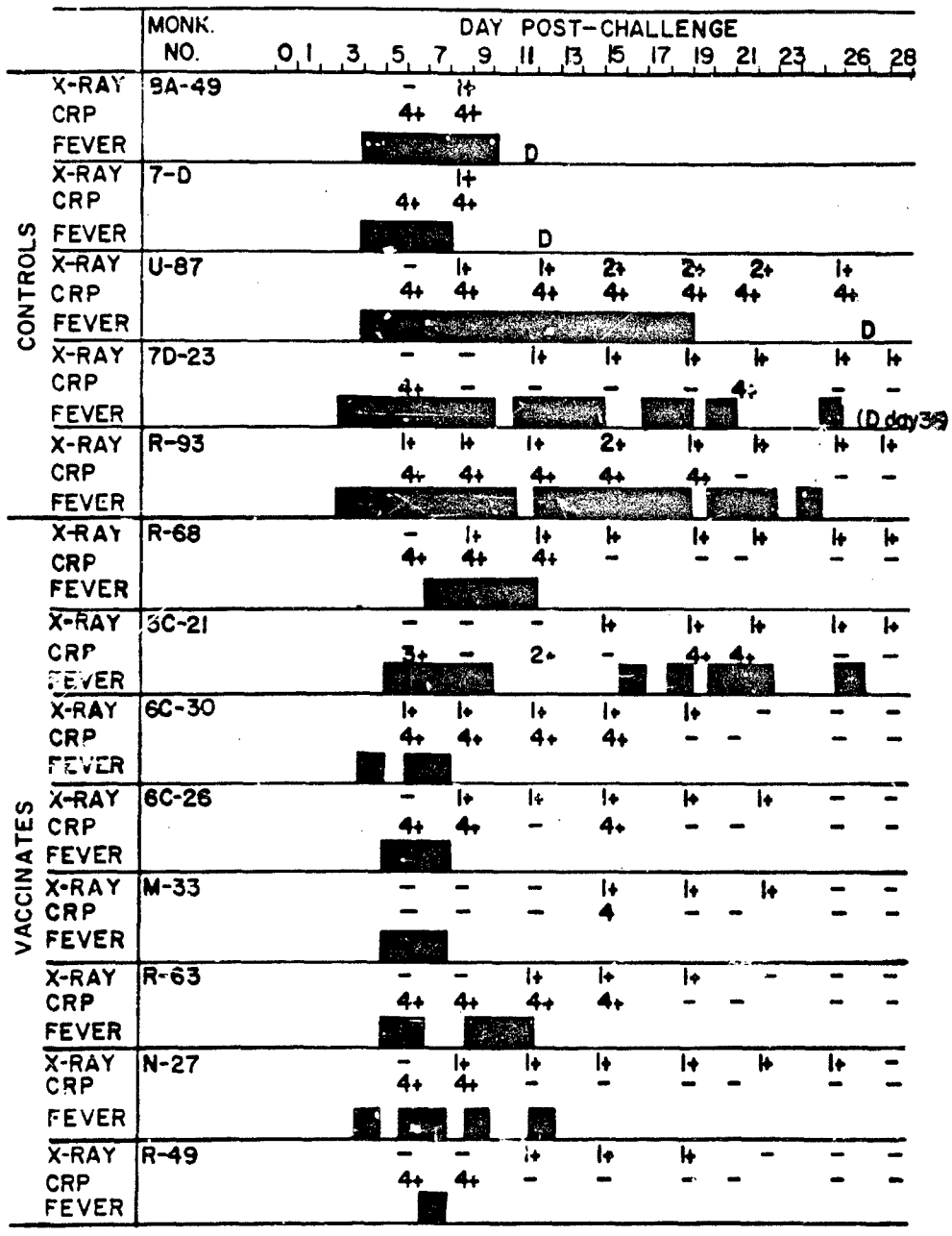

FIGURE 4. RESPONSES OF *M. MULATTA* TO AEROSOLIZED *P. TULARENSIS* SCHU – 35, HIGH DOSE (4500 CELLS).

Summary and Conclusions:

Studies were conducted to evaluate the efficacy of LVS in protecting monkeys against respiratory challenge of SCHU-S4 and SCHU-S5 strains of P. tularensis. These studies do not establish whether or not there is a difference in protection afforded against strains SCHU-S4 and SCHU-S5, but do indicate a need for extending the observations to larger numbers.

Publications:

None.

ABSTRACT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-02:	Prevention and Treatment of BW Casualties
Study No. -00-02:	Evaluation of Efficacy of Experimental Vaccines.
	Section II: Determination of Human Median Infectious Dose of Attenuated VEE Virus (ND 4-93)
Reporting Installation:	U. S. Army Medical Unit Fort Detrick, Maryland
Division:	Medical
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Authors:	William D. Sawyer, Major, MC Thomas J. Smith, Major, MC Robert W. McKinney, Major, MSC
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

Attenuated Venezuelan equine encephalomyelitis virus (National Drug Lot 4-93rd passage) was titrated in a group of volunteers. Forty-two subjects were inoculated subcutaneously with serial 10-fold dilutions of the virus. It was determined that this lot of vaccine contained 3.46×10^4 human ID_{50}/0.5 ml. Simultaneous titration in guinea pigs indicated that the preparation contained 7.4×10^5 $GPIPID_{50}$/0.3 ml. Of 18 individuals responding serologically to the vaccine, virus was recovered from throat washings of 6, and the blood of one; clinical reactions occurred in 12 of the 18.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-02: Prevention and Treatment of BW Casualties

Study No. -00-02: Evaluation of Efficacy of Experimental Vaccines

Section II: Determination of Human Median Infectious Dose of Attenuated VEE Virus (ND 4-93)

Description:

To evaluate experimental vaccines developed by various organizations, contractors, or other governmental agencies.

Progress:

Forty-two volunteers who had no known immunological experience with Venezuelan equine encephalomyelitis (VEE) were inoculated subcutaneously with serial 10-fold dilutions of attenuated VEE virus (National Drug Lot 4-93rd passage). The men were divided into 7 dosage groups of 6 men each. It had been anticipated that dosage of virus would range from 100,000 to 0.1 guinea pig intraperitoneal median infectious doses ($GPIPID_{50}$). On the basis of simultaneous titration of the virus in guinea pigs, however, dosage in volunteers ranged from 7,400 to 0.007 $GPIPID_{50}$.

The men were observed employing double-blind technique for 12 days following inoculation and all physical signs and symptoms recorded. Attempts at virus isolation from blood and pharyngeal washings were made on 3 occasions in each man during the 12-day period. Three-tenths milliliter of each specimen was inoculated IP into each of 6 weanling white mice; presence of the attenuated virus was demonstrated by survival of mice after challenge with virulent VEE virus 14 days later. Hemagglutination-inhibiting (HAI) antibody titers were determined on sera obtained prior to virus administration and on days 14, 28, and 56 postinoculation. (Table I) All 6 individuals in the highest dosage group responded serologically, although some did so only to a minimal degree. One man in the 2nd group showed no change in titer, while 2 in the 3rd group demonstrated no rise in titer. Half of the 4th group showed a serologic response, while none in the remaining groups were immunized.

There were a total of 12 reactions to the vaccine administration. The most common manifestations were headache, fever, myalgia, eye pain, fatigue and weakness. Conjunctivitis was the most common abnormal physical finding. A mild leukopenia with a relative lymphocytosis was noted commonly. All reactions were short-lived and there were no sequelae.

TABLE I. HAI RESPONSE[a] IN HUMANS VACCINATED WITH ATTENUATED VEE VIRUS (ND-4).

ESTIMATED DOSE ($GPIPID_{50}$)	SUBJECTS	RECIPROCAL HAI TITER[b]		
		Day 14	Day 28	Day 56
7400	1	10	10	<10
	2	<10	10	<10
	3	320	320	80
	4	40	80	40
	5	20	40	40
	6	20	20	20
740	1	20	10	<10
	2	<10	<10	<10
	3	10	20	20
	4	40	80	80
	5	20	80	80
	6	80	160	80
74	1	<10	<10	<10
	2	80	160	160
	3	40	80	80
	4	<10	20	20
	5	<10	<10	<10
	6	<10	160	80
7.4	1	20	80	80
	2	<10	<10	<10
	3	<10	<10	<10
	4	<10	40	20
	5	<10	40	40
	6	<10	<10	<10

a/ 0.74, 0.07, and 0.007 $GPIPID_{50}$ doses of live virus resulted in no HAI response in each of 6 individuals.

b/ Pre-immunization titer <10.

Virus was isolated from throat washings of 6 individuals, while viremia was noted in only one. These 6 subjects were distributed throughout the 4 groups receiving higher vaccine doses. Postinoculation day 6 was the earliest virus was detected, and day 12 was the latest. The volunteer in whom viremia was detected had a severe reaction of a biphasic nature, requiring hospitalization on postinoculation day 6 because of high fever, headache, myalgia, anorexia, nausea, vomiting and abdominal pain. He became well in several days. Viremia was present on days 6 and 7, while throat washings contained virus on days 6-10. A lumbar puncture performed on day 6 revealed a cell-free spinal fluid with normal protein and sugan content. No virus was isolated from spinal fluid.

Table II depicts the number of individuals responding serologically in each dosage group, the number of reactions within each group, and the results of virus isolation attempts.

TABLE II. RESULTS OF TITRATION OF ATTENUATED VEE VIRUS (ND 4-92rd PASSAGE) IN VOLUNTEERS (6 AT EACH DOSE LEVEL).

DOSE OF VACCINE ADMINISTERED[a] ($GPIPID_{50}$)	NO. WITH SEROLOGIC RESPONSE	NO. OF REACTIONS	NO. WITH VIRUS IN THROAT WASHINGS	NO. WITH VIREMIA
7400	6	4	1	0
740	5	3	2	0
74	4	3	2	1
7.4	3	2	1	0

a/ Volunteers given 0.74, 0.07, and 0.007 $GPIPID_{50}$ were nonreactive in all 4 categories.

On the basis of HAI response in this group of volunteers the vaccine contained 3.46×10^4 human subcutaneous ID_{50}/0.5 ml. Simultaneous guinea pig titration indicated that the preparation contained 7.4×10^5 $GPIPID_{50}$/0.5 ml. Thus, infectivity for man was less than 1/10 that for guinea pigs. Since IP and subcutaneous inrectivity of VEE virus for guinea pigs is similar the interspecies difference noted here is thought not to result from the difference in route of administration.

Summary:

A titration of attenuated VEE virus (ND 4-93rd passage) was conducted in volunteers. The human subcutaneous ID_{50} was found to be more than 10 times the guinea pig $IPID_{50}$. The incidence of significant reactions to the vaccine was high, with 12 of 18 who responded serologically experiencing symptoms.

Publications:

None.

ANNUAL PROGRESS REPORT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-02:	Prevention and Treatment of Biological Warfare Casualties
Study No. -00-03:	Chemoprophylaxis and Therapy of Infectious Diseases of Potential Biological Warfare Significance.
Reporting Installation:	U. S. Army Medical Unit Fort Detrick, Maryland
Divisions:	Medical and Animal Assessment
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Authors:	Harry G. Dangerfield, Major, MC, Sect. I William C. Day, Captain, VC, Sect. I Ralph W. Kuehne, Sect. I, II, [redacted] James W. Higbee, Lietenant, MSC, Sect. I Arthur L. Hogge, Jr., Colonel, VC, Sect. I William D. Sawyer, Major, MC, Sect. I, II, III Robert E. Nopar, Captain, MC, Sect. II, III Sheldon Sidell, Captain, MC, Sect. II, [redacted] Dan Crozier, Colonel, MC, Sect. II, III
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-02:	Prevention and Treatment of Biological Warfare Casualties
Study No. -00-03:	Chemoprophylaxis and Therapy of Infectious Diseases of Potential Biological Warfare Significance.
	Section I: Postexposure Treatment of Airborne Simian Tularemia with Tetracycline, Novobiocin, Kanamycin, and Gentamicin.
Reporting Installation:	U. S. Army Medical Unit Fort Detrick, Maryland
Divisions:	Medical and Animal Assessment
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Authors:	Harry G. Dangerfield, Major, MC William C. Day, Captain, VC Ralph W. Kuehne James W. Higbee, Lieutenant, MSC Arthur L. Hogge, Jr., Colonel, VC William D. Sawyer, Major, MC
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

An in vitro evaluation of the sensitivity of Pasteurella tularensis, SCHU-S5 strain, to ampicillin, gentamicin, kanamycin, and novobiocin was made by quantitative tube dilution studies. Of the drugs tested ampicillin was found to be ineffective. The results obtained indicate that novobiocin was bacteriostatic while kanamycin and gentamicin were bactericidal for the streptomycin-resistant strain of P. tularensis.

Studies were conducted to determine if these observations could be confirmed in rhesus monkeys challenged with aerosolized SCHU-S5 P. tularensis. Tetracycline, novobiocin, gentamicin, and kanamycin were the therapeutic regimens employed. In contrast to the former antibiotics, kanamycin was found to be bactericidal and its efficacy in treating streptomycin-resistant P. tularensis appears comparable to that achieved by streptomycin against SCHU-S4 P. tularensis.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-02: Prevention and Treatment of Biological Warfare Casualties

Study No. -00-03: Chemoprophylaxis and Therapy of Infectious Diseases of Potential Biological Warfare Significance.

Section I: Postexposure Treatment of Airborne Simian Tularemia with Tetracycline, Novobiocin, Kanamycin, and Gentamicin.

Description:

To assess the effect of selected antimicrobial drugs and of various drug regimens for prophylaxis and therapy of infectious diseases.

Section I. To develop an effective schedule of treatment of simian tularemia.

Progress:

During the current reporting period, the antibiotic sensitivity of P. tularensis, SCHU-S5 strain, to kanamycin, novobiocin, ampicillin (supplied by Bristol Laboratories), and gentamicin (supplied by Schering Corporation) has been evaluated utilizing the tube dilution technique of Eigelsbach, Giesken, and Halstead (MB Div, Quarterly Report, USABL, 31 Mar 1957).

Serial 1:2 dilutions of each antibiotic, including streptomycin, were inoculated with a known concentration of SCHU-S5 P. tularensis (supplied by Dr. H. T. Eigelsbach, MB Division, USABL). These antibiotic concentrations encompass a range which is easily achieved in human serum. All cultures were incubated at 37 C; at 12, 24, 48, and 72 hr, quantitative viable counts were made in triplicate on plates inoculated with serial dilutions of each antibiotic concentration. Liquid and semi-solid "T" media (Tresselt and Ward, USAMU Annual Progress Report, FY 1963, Task No. 03-10-03) were employed throughout.

The results of these in vitro studies are summarized in Table I. By comparing the relationship of growth of organisms in the various concentrations of antibiotics an evaluation can be made regarding the bacteriostatic or bactericidal effect of a given antibiotic. These data indicate that novobiocin is bacteriostatic while kanamycin and gentamicin are bactericidal for the streptomycin-resistant strain of P. tularensis (Table I).

TABLE I. IN VITRO SENSITIVITY OF P. TULARENSIS SCHU-S5 TO VARIOUS ANTIBIOTICS

ANTIBIOTIC	SCHU-S5 INOCULUM $\log_{10}$	HOURS INCU-BATION	NO. OF ORGANISMS (LOG_{10}) AT ANTIBIOTIC CONCENTRATION (μg/ml)											
			0	0.38	0.75	1.5	3.1	6.25	12.5	25	50	100	500	1000
Streptomycin	5.4	24	8.5			8.3	8.3	8.3	7.9	7.5	5.7	7.4	8.4	8.2
		48	8.1			8.4	8.6	8.8	8.7	8.9	8.9	7.7	8.5	8.5
		72	8.2			7.7	8.3	8.5	8.5	8.7	8.3	8.4	8.5	8.2
Ampicillin	4.5	12	4.9	4.8	4.6	4.8	4.2	4.0	3.8	3.1	2.9	2.9		
		24	7.5	6.5	6.4	6.7	7.8	4.8	4.4	3.4	2.9	2.4		
		48	8.8	8.8	7.5	8.6	4.7	3.2	2.0	0[a]	0	0		
		72	9.5	9.5	9.3	8.2	4.4	2.3	0	0	0	0		
Gentamicin	3.1	12	4.8	1.7	1.0	0	0	0	0	0	0	0		
		24	6.1	0	0	0	0	0	0	0	0	0		
		48	9.2	0	0	0	0	0	0	0	0	0		
		72	10.2	0	0	0	0	0	0	0	0	0		
Novobiocin	4.3	12	5.7			2.9	2.8	1.9	0					
		24	6.9			2.2	1.7	0	0					
		48	7.2			0	0	0	0					
		72	8.9			0	0	0	0					
Kanamycin	7.4	12	8.6			5.1	4.8	4.2	4.1					
		24	8.2			0	0	0	0					
		48	8.1			0	0	0	0					
		72	8.4			0	0	0	0					

a. No growth

Based on these findings, experiments were designed to compare the efficacy of kanamycin, gentamicin, novobiocin, and tetracycline (the current drug of choice) in the therapy of simian infection by the SCHU-S5 strain of P. tularensis.

Forty-eight young adult Macaca mulatta, weighing 2-3 kg were exposed to aerosols of P. tularensis (SCHU-S5) employing a modified Henderson apparatus. The challenge doses ranged from 3040 cells to 10,160 cells. These monkeys were challenged at 2 different times. The first study was accomplished to determine the therapeutic efficacy of novobiocin using 2 different regimens of therapy; the second study was designed to compare the effects of tetracycline, kanamycin, and gentamicin against the streptomycin-resistant strain of P. tularensis.

In the novobiocin study, 18 monkeys were randomly divided into 3 groups of 6 animals each. Two nonexposed monkeys served as drug controls; and were given 45 mg of novobiocin orally 3 times a day, at 0800, 1600 and 2400 hr for 17 days. Challenged monkeys were started on therapy, drug regimen as above, immediately following 2 consecutive rectal temperatures of 104.0 F or one temperature of 106.0 F or greater in Group I and 72 hours after a significant fever, as defined above, in Group II. Group III was not treated.

Therapy, once initiated, was continued for a minimum of 21 doses. Prior to cessation of treatment, animals were required to be afebrile for at least 72 hours. Animals relapsing were treated secondarily for at least 72 hr following cessation of fever. Further relapses were not treated.

Studies on individual animals included temperatures twice daily, chest radiographs on days 3, 6, 9, 14, and 21, WBC, CRP, and daily weight determinations. All animals were bled for serology on days 7, 14, 21, and 28, the day of sacrifice.

Liver, spleen, blood and brain of animals dying during the study and survivors at time of sacrifice were cultured for P. tularensis. Necropsy results will be reported by Pathology Division, USAMU.

Untreated control animals (Figure 1) developed fever by day 3 and remained febrile until death. Chest x-rays showed moderate pulmonary infiltrates in all monkeys by day 6. Positive CRP's appeared at the same time or slightly earlier than fever and remained positive until death. All specimens cultured at necropsy were positive for P. tularensis.

All animals in Group I developed fever by day 3 (Figure 2). Novobiocin was initiated on the same day. Four animals received 21 doses, 1 received 28, and another 51. Following institution of therapy, 4 animals were afebrile within 72 hr; however, 2 continued with intermittent fever throughout the course of therapy. All CRP's were positive by day 3 and negative by day 8. Five of 6 animals had positive chest x-rays by day 6; the last became positive on day 9.

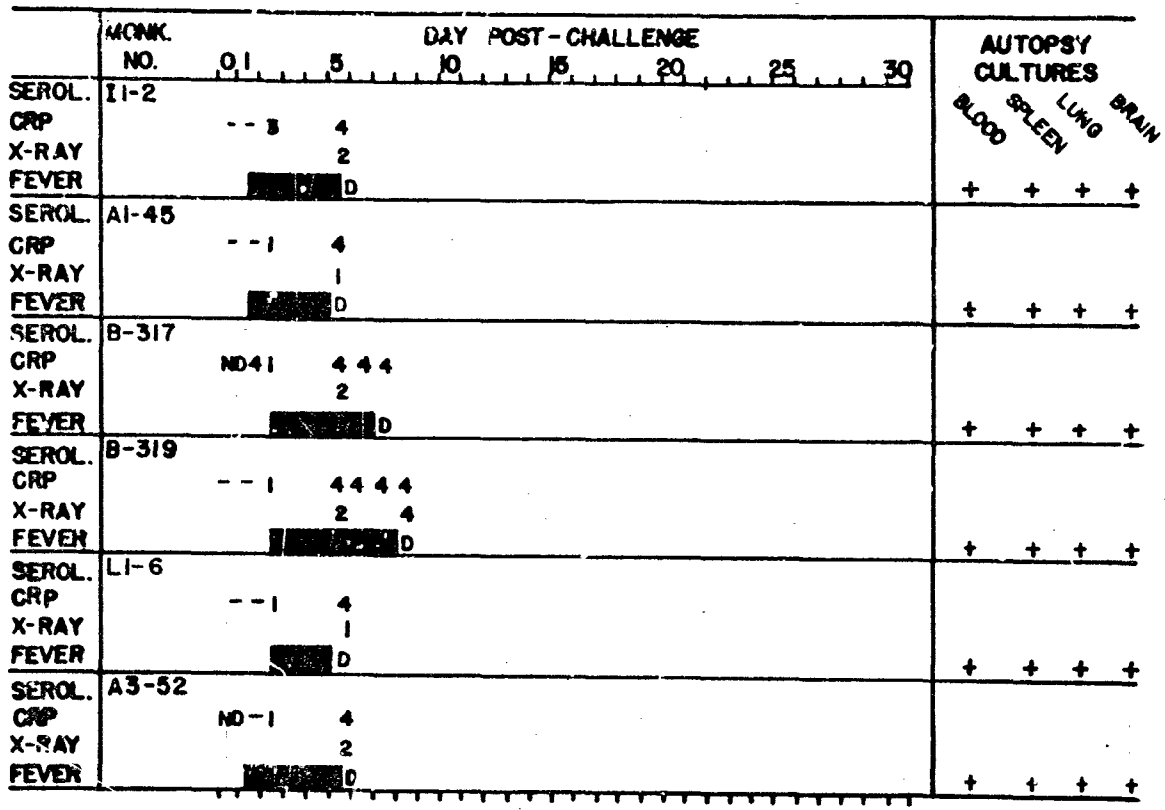

FIGURE I. RESPONSE OF UNTREATED *M. MULATTA* TO *P. TULARENSIS*, SCHU-S5.

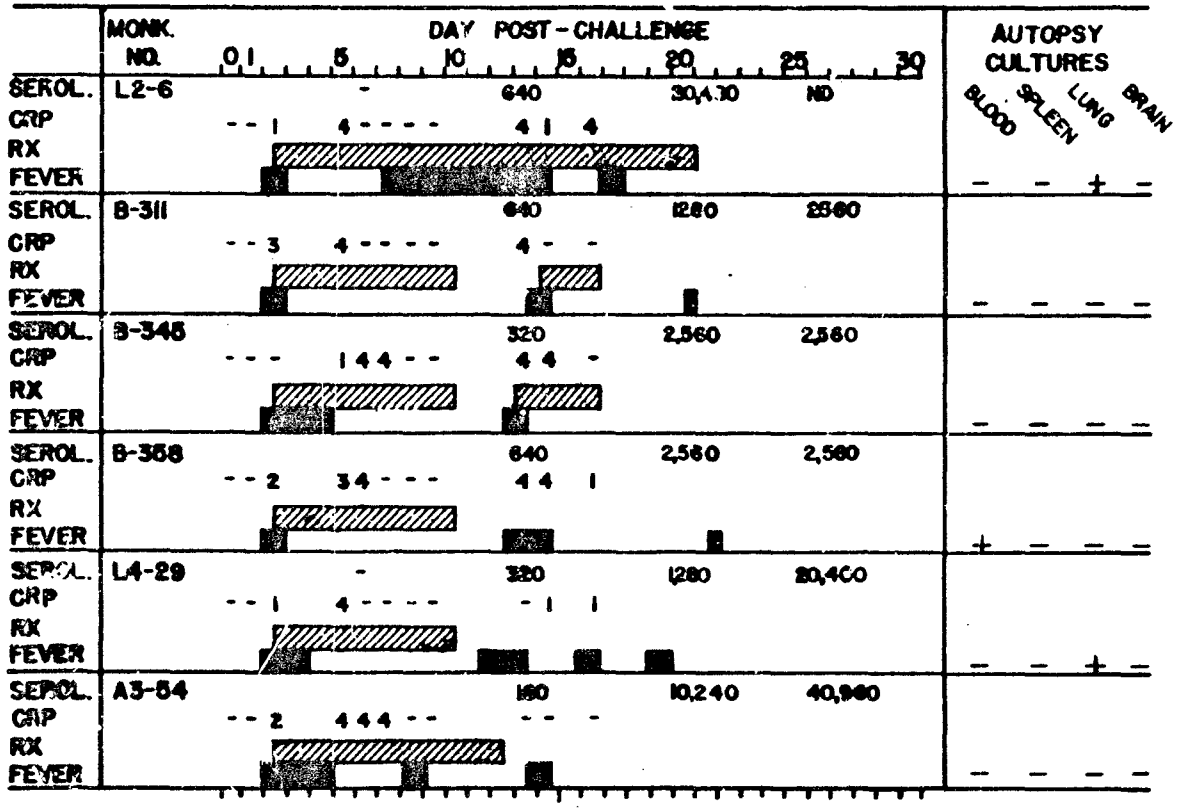

FIGURE 2. RESPONSE OF TREATED *M. MULATTA* TO *P. TULARENSIS*, SCHU-S0., NOVOBIOCIN, ORAL, 45mg, 3X DAILY, STARTED AFTER FIRST SIGNIFICANT FEVER, X 21 DOSES AND CONTINUING 72 HOURS AFEBRILE.

Clinical relapse, as evidenced by recurrence of fever, occurred in 5 of 6 animals following cessation of treatment. Two of the 5 were retreated and each relapsed again following cessation of therapy. P. tularensis was recovered from the lungs of 2 monkeys and from blood of another at the time of sacrifice.

Novobiocin was delayed until 72 hr following a significant temperature elevation (vide supra) in animals comprising Group II. The monkeys were febrile by day 2 and therapy was begun on day 6 (Figure 3). One animal died after only 2 doses of novobiocin, another received 24 doses and the remaining 4 animals received 21 doses. Following completion of therapy febrile relapses occurred in the 5 surviving monkeys. Two were retreated but again relapsed when novobiocin was stopped. One of the 3 untreated relapsed animals succumbed to the infection. Extensive pulmonary infiltrates were seen in all survivors by day 6. Cultures taken from animals dying during the course of the study were all positive for P. tularensis whereas positive cultures were found in only 2 of the 4 survivors at time of sacrifice. The organism was recovered from the lungs of each and in one from brain and spleen as well.

In the second study, kanamycin and gentamicin were evaluated in the therapy of streptomycin-resistant P. tularensis infection. The efficacy of these antibiotics was compared with the current drug of choice, tetracycline.

Thirty-six M. mulatta were employed in this study and were grouped as follows:

Group A - 6 animals as untreated controls.
Group B - 6 animals as drug controls (2/antibiotic)
Group C - 8 animals treated orally 3 times a day with 75 mg of tetracycline
Group D - 8 animals treated IM 3 times daily with 3 mg of gentamicin
Group E - 8 animals treated as in Group D with 30 mg of kanamycin

The criteria for initiation and stopping therapy with these drugs were the same as those employed in the novobiocin study. Drugs were administered at 0800, 1600, and 2400 hr. Dose levels employed were given on a weight basis to achieve therapeutic levels comparable to those required for man.

The untreated control animals (Group A) responded in a similar manner to those shown in Figure 1 (novobiocin study). Adverse effects were not observed in those animals serving as drug controls (Group B).

Postchallenge observations included temperatures twice daily, weekly chest roentgenograms, and serologic response at 7-day intervals. Attempts were made to recover P. tularensis from animals that died during the study as well as those sacrificed 28 days postchallenge.

The tetracycline-treated monkeys (Group C) (Figure 4) were febrile by day 3 at which time therapy was initiated. Within 48 hours all were afebrile and with the exception of 1 animal remained so during the course of treatment. Clinical relapses occurred in all monkeys within 5 days following completion

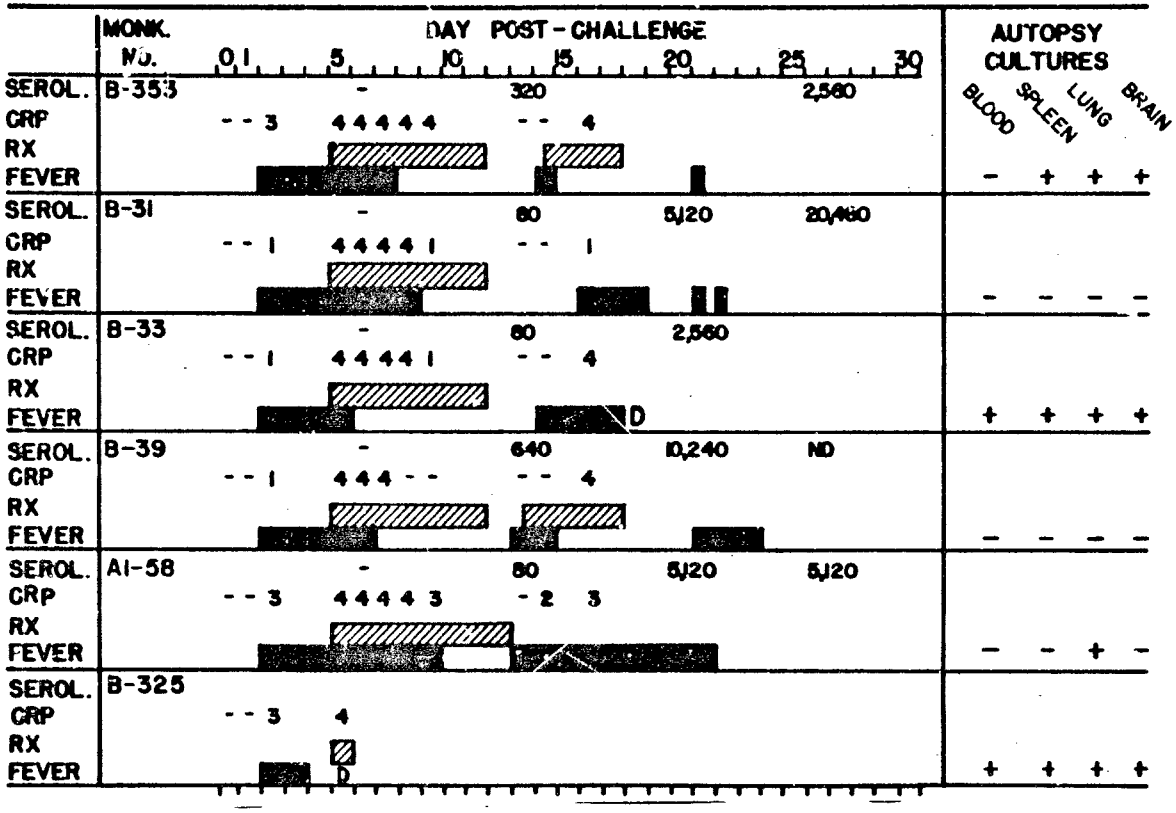

FIGURE 3. RESPONSE OF TREATED *M. MULATTA* TO *P. TULARENSIS*, SCHU-S5. NOVOBIOCIN, ORAL, 45mg, 3X DAILY, STARTED 72 HOURS AFTER FIRST SIGNIFICANT FEVER, X 21 DOSES AND CONTINUING 72 HOURS AFEBRILE.

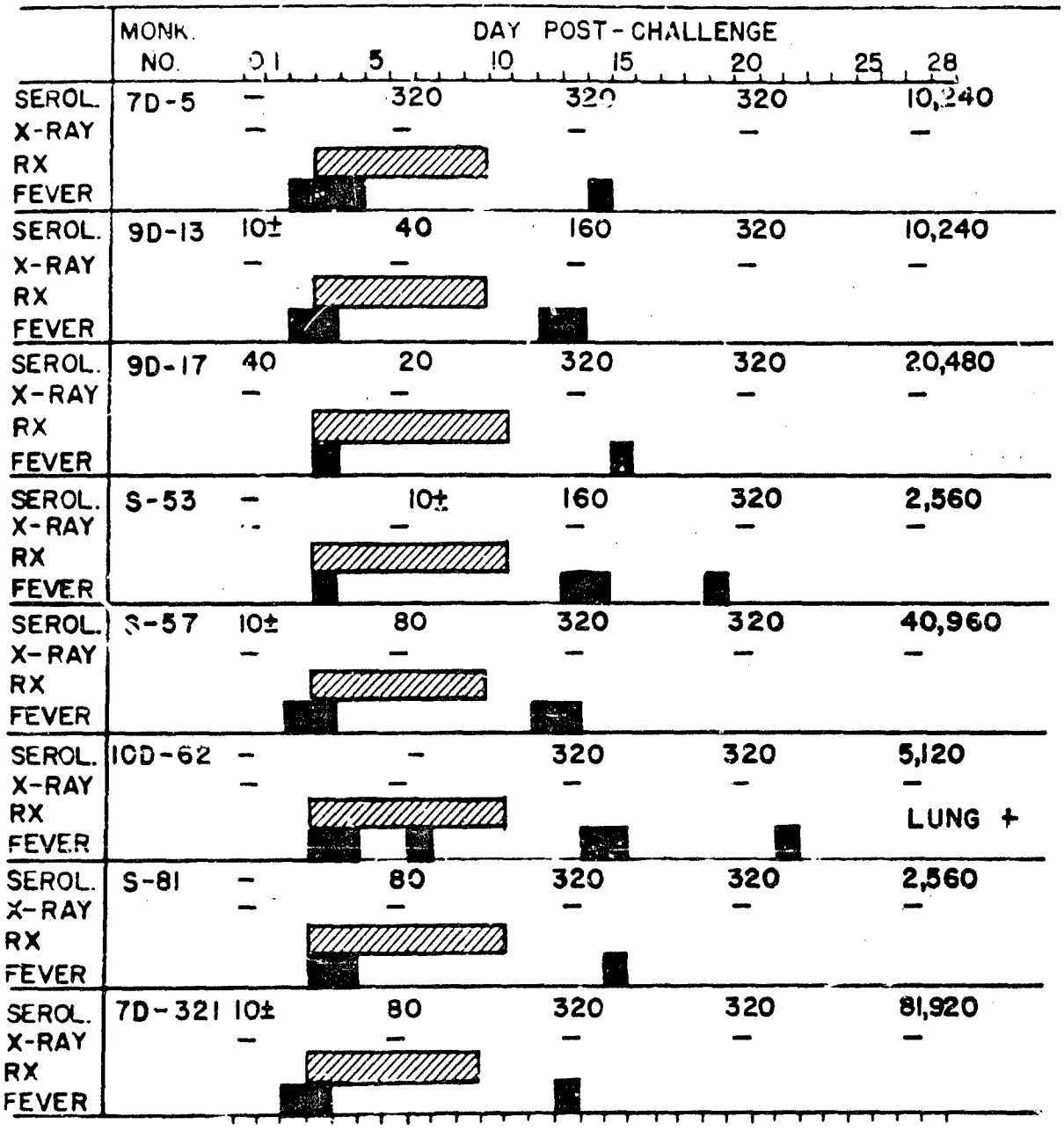

FIGURE 4. RESPONSE OF TREATED *M. MULATTA* TO *P. TULARENSIS*, SCHU-S5 TETRACYCLINE, ORAL, 45mg, 3X DAILY, STARTED AFTER FIRST SIGNIFICANT FEVER, X 21 DOSES, AND CONTINUING 72 HOURS AFEBRILE

of the therapeutic regimen. Radiographic evidence of pulmonary involvement was not seen. No deaths occurred; however, P. tularensis was isolated from the lung of 1 animal at time of sacrifice.

The monkeys that received gentamicin (Group D) (Figure 5) became febrile at the same time as those treated with tetracycline. In contrast to the tetracycline-treated group, fever persisted from 3-5 days following the onset of treatment. Radiographic examination of the chest revealed pulmonary infiltration in 6 of the 8 animals. Seven of 8 animals experienced clinical relapse following cessation of gentamicin therapy. None of the animals died and when sacrificed on day 28 P. tularensis could not be recovered.

The efficacy of kanamycin in treating streptomycin-resistant simian tularemia appears comparable.

Animals in Group E, kanamycin therapy were also ill by day 3 as illustrated in Figure 6. Therapy was initiated at this time. All animals were afebrile after 48 hr treatment. Although a low grade fever occurred in 2 animals during treatment, clinical relapses did not occur in any kanamycin-treated animal. With the exception of 1 monkey, all chest x-rays were within normal limits during the study. On day 6 postchallenge the radiograph of this monkey revealed a moderate pulmonary infiltration. All animals survived and were sacrificed on day 28. All cultures were negative for P. tularensis.

Summary:

An in vitro evaluation of the sensitivity of P. tularensis, SCHU-S5 strain to ampicillin, gentamicin, kanamycin, and novobiocin was made by quantitative tube dilution studies. Of the drugs tested ampicillin was found to be ineffective. The results obtained indicated that novobiocin was bacteriostatic while kanamycin and gentamicin were bactericidal for the streptomycin-resistant strain of P. tularensis.

Studies were conducted to determine if these observations could be confirmed in rhesus monkeys challenged with aerosolized SCHU-S5 P. tularensis. Tetracycline, novobiocin, gentamicin, and kanamycin were the therapeutic regimens employed. In contrast to the former antibiotics, kanamycin was found to be bactericidal and its efficacy in treating streptomycin-resistant P. tularensis appears comparable to that achieved by streptomycin against SCHU-4 P. tularensis.

Publications:

None.

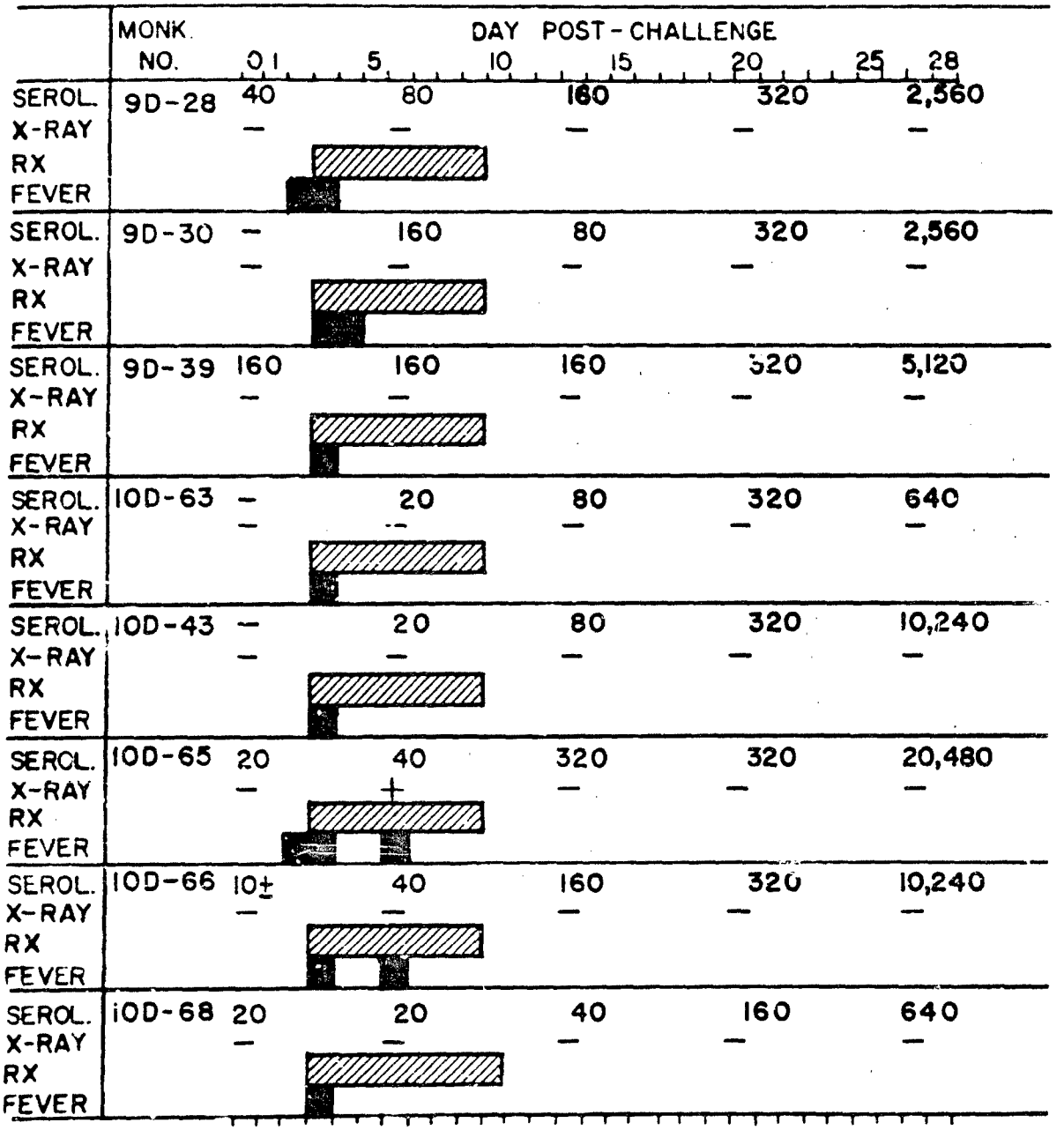

FIGURE 5. RESPONSE OF TREATED *M. MULATTA* TO *P. TULARENSIS*, SCHU-S5. GENTAMYCIN, IM, 3mg, 3X DAILY, STARTED AFTER FIRST SIGNIFICANT FEVER, X 21 DOSES AND CONTINUING 72 HOURS AFEBRILE.

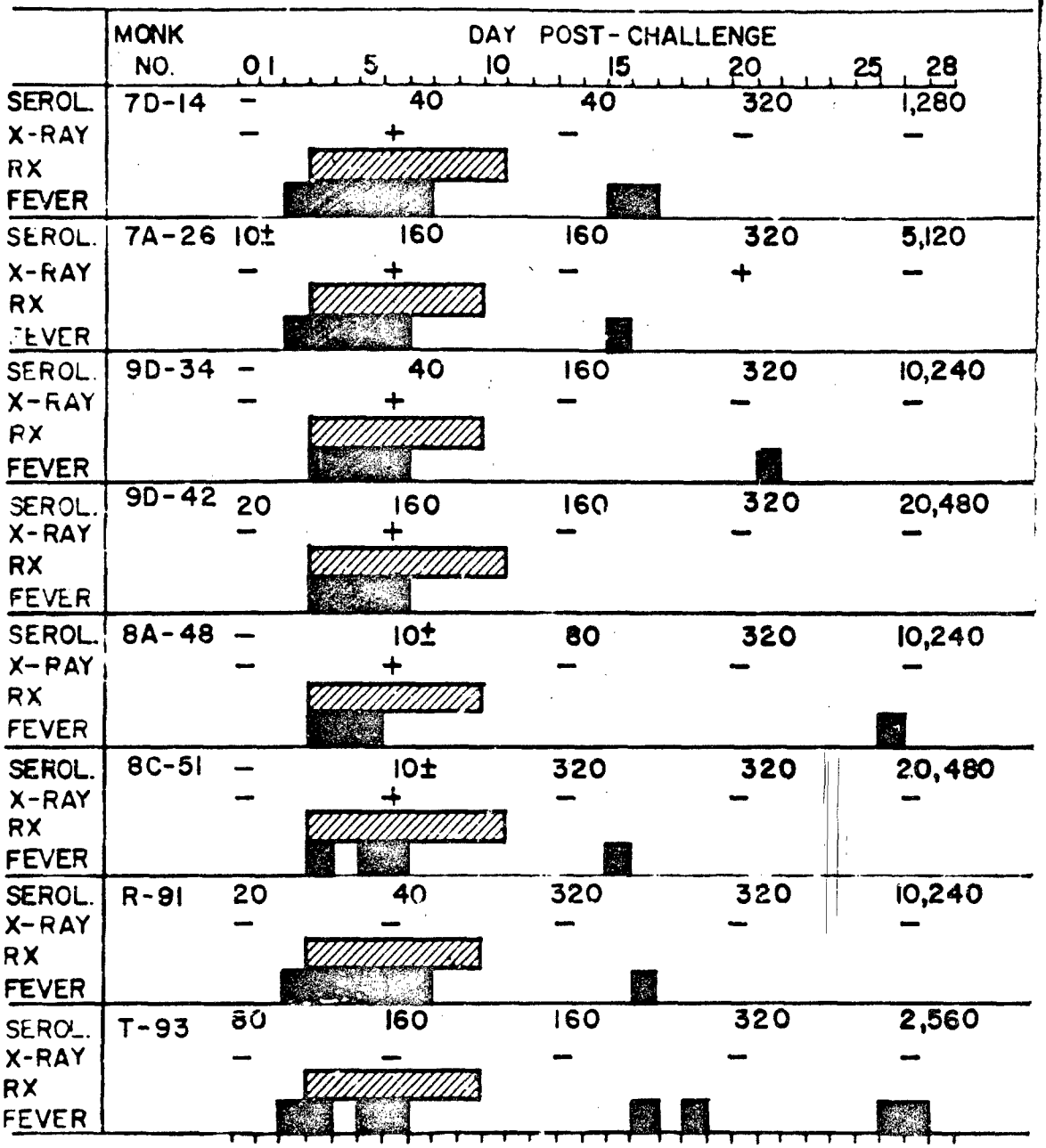

FIGURE 6. RESPONSE OF TREATED *M. MULATTA* TO *P. TULARENSIS*, SCHU-S5. KANAMYCIN, IM, 30mg, 3X DAILY, STARTED AFTER FIRST SIGNIFICANT AND CONTINUING 72 HOURS AFEBRILE.

ABSTRACT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-02:	Prevention and Treatment of Biological Warfare Casualties
Study No. -00-03.	Chemoprophylaxis and Therapy of Infectious Diseases of Potential Biological Warfare Significance.
	Section II. Tetracycline Treatment and Post-exposure Prophylaxis of Experimental Human Respiratory Tularemia.
Reporting Installation:	U. S. Army Medical Unit Fort Detrick, Maryland
Division:	Medical
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Authors:	William D. Sawyer, Major, MC Sheldon Sidell, Captain, MC Ralph W. Kuehne Robert E. Nopar, Captain, MC Dan Crozier, Colonel, MC
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

Interrupted and continuous courses of tetracycline have been evaluated in the treatment and postexposure prophylaxis of experimental respiratory tularemia in volunteers. For treatment an interrupted course of tetracycline was effective in achieving and maintaining well-being without later major symptomatic relapse. This regimen of 4.0 gm in divided doses on day 1 followed by 4 days of 0.5 gm each 6 hr; 3 days without drug; 5 days of 0.5 gm each 6 hr; another 3 days off drug; and finally 0.5 gm at 6-hr intervals for 5 additional days. Variations of this schedule and continuous treatment for 10 days were noticeably deficient either because of illness between courses of treatment or by relapse following treatment.

Continuous administration of tetracycline, 0.5 gm twice daily, for 28 and for 42 days prevented later onset of tularemia, but 20% of subjects were more or less disabled for a short time by symptoms, possibly related to tularemia, during the course of tetracycline. Intermittent tetracycline, 0.5 gm twice on every other day over a 19-day period, failed to prevent

subsequent development of overt illness.

An unusual incidence, 4 of 8 subjects, of photoallergy presumably related to tetracycline followed one particular interrupted schedule of administration.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-02: Prevention and Treatment of Biological Warfare Casualties

Study No. -00-03: Chemoprophylaxis and Therapy of Infectious Diseases of Potential Biological Warfare Significance

Section II. Tetracycline Treatment and Post-exposure Prophylaxis of Experimental Human Respiratory Tularemia.

Description:

To assess the effect of selected antimicrobial drugs and of various drug regimens for prophylaxis and therapy of infectious diseases.

Section II. To develop an effective schedule of tetracycline treatment and postexposure prophylaxis of human respiratory tularemia.

Progress:

Broad spectrum antibiotics are effective for treatment of acute tularemia. Their use is complicated, however, by frequent relapses following cessation of treatment, especially, it is said, when treatment is instituted early in the illness. When employed during the incubation period short courses of broad spectrum drugs do not prevent disease but simply delay its onset for the period of treatment. Both phenomena apparently are related to the bacteriostatic effect of these drugs upon Pasteurella tularensis; organism multiplication is suppressed and bacterial numbers remain beneath the threshold for clinical disease but are not eliminated from the host by antibiotic action. In tularemia, as in other intracellular parasitic infections such as scrub typhus, Q fever and typhoid, the capacity of host mechanisms to clear the parasitic burden is limited, especially early in disease. Last year's studies in monkeys described efforts to adapt concepts of intermittent administration of bacteriostatic agents which have proven efficacious in other examples of intracellular parasitism to tularemia (USAMU Annual Progress Report, FY 1963). The efforts were not completely successful. The best of the regimens were, however, considered sufficiently encouraging to warrant extension of the studies to man with some modifications of schedule of drug administration. The work on both prophylaxis and therapy during the reporting period was performed in 2 separate experiments; a third study is in progress and will be discussed only in terms of its relation to completed work.

Initally 2 separate programs of prophylaxis were examined. Sixteen

volunteers inhaled 27,000 (range 24,000-34,000) viable cells of P. tularensis, SCHU-S4 strain, employing a modified Henderson apparatus. Beginning 24 hr after exposure half received 0.5 gm tetracycline twice daily for 42 days. The remaining 8 began at the same time a course consisting of 0.5 gm tetracycline twice on odd numbered days, i.e. 1, 3, 5, etc. for 19 days. To make the results more meaningful in terms of ability to perform duties all volunteers worked, unless symptomatic, at their normal military assignment throughout the prophylaxis period.

Of men receiving drug every other day, 5 escaped illness during the period of tetracycline administration but 3 volunteers experienced symptoms and fever. One man had fever to 101 F and moderate symptoms compatible with typhoidal tularemia days 5-7 but then returned to well-being. A second was similarly affected (fever to 102 F) days 6-8. These two men missed work during the period of symptoms. The third subject worked throughout the period of prophylaxis but comparison of his temperature curve with that before and after suggests he may have had very low grade fever (100 to 100.4 F) during the course of tetracycline. Following cessation of tetracycline 7 men developed typical typhoidal tularemia (5 on day 3 off drug and 2 on day 4). They were sufficiently ill to require treatment, in this case with streptomycin. The 8th subject had no fever but complained repeatedly of retrosternal chest pain. Chest films and efforts to isolate P. tularensis were negative. Erythrocyte sedimentation rates were borderline elevated. Finally on the 23rd day off tetracycline, he was begun on streptomycin with rapid remission of symptoms. Bacterial agglutinins were present in the serum of all subjects, though in low titer (range 1:10 to 1:80), at the time tetracycline was terminated. Titers further increased in all to 1:80 to 1:640, 6 weeks after exposure.

Daily administration of tetracycline for 42 days proved more successful as a prophylactic program. Two subjects experienced low grade, non-specific symptoms, lassitude, vague myalgia, malaise, and mild cephalalgia, together with flattening of diurnal temperature variation for 3 and 8 days during the course of tetracycline. One didn't feel like working for 3 of the 8 days he had such symptoms. The remaining men remained completely well. Following cessation of tetracycline no evidence of illness was detected in any of the volunteers. Bacterial agglutinins were not detected at any time in sera from 7 of these subjects, including the man experiencing symptoms. This latter observation perhaps added support to the clinical feeling at the time that the symptoms during tetracycline treatment were unrelated to tularemia. One man had a low agglutinin titer (1:10) 2 weeks following exposure which remained essentially unchanged 9 weeks later.

The success of the 42-day prophylaxis led to testing of a shorter schedule, 28 days in the second set of studies. The inhaled dose of P. tularensis was similar to that of Phase I, 23,000 (range 17,600 to 28,600) viable cells. One of the 8 volunteers stated he was not feeling completely well on days 8, 9, and 13-15. These symptoms were associated with loss of diurnal temperature variation but his peak temperatures were

not elevated; the other 7 men remained well. All 8 men remained completely well after tetracycline was discontinued. Serological studies are in progress.

An examination of the efficacy of a 14-day course of tetracycline prophylaxis, 0.5 gm twice daily beginning 24 hr after exposure is in progress. It is notable that one volunteer had headache, myalgia and malaise compatible with typhoidal tularemia accompanied by fever (to 101.4 F) from days 7-11 while receiving tetracycline.

These results together with those obtained employing monkeys imply that postexposure prophylaxis of respiratory tularemia with broad spectrum antibiotics is of but limited practical value. Considerable quantities of drug and relatively prolonged courses seem to be necessary. Moreover, symptoms sufficient to reduce work capacity occurred in 20% (4 of 20) of the volunteers during the time they were receiving daily tetracycline; it is not certain, however, whether these were incidental or related to tularemia. It is worth considering in the event of potential exposure that in many cases it would be perhaps more efficient in terms of drug, time and trouble to allow illness to develop and to begin treatment early, before disability becomes significant.

The initial phase of evaluation of tetracycline therapy involved only 6 subjects. Inhaled doses were 27,000 viable cells of P. tularensis, SCHU-S4. After illness was well established, i.e. 12 to 24 hr of fever and typical symptomatology, they were divided at random into 2 treatment groups. Three men were treated with 4 gm tetracycline in divided doses the first day followed by 0.5 gm every 6 hours for 9 additional days. Initial response was excellent. Relapses of sufficient severity to require therapy occurred, however, in each beginning 5, 7 and 8 days after termination of tetracycline.

The other 3 men as well as 7 men from the second experiment (inhaled dose of P. tularensis was 23,000 viable cells) received interrupted tetracycline treatment: 1 gm every 6 hr the first day then 0.5 gm every 6 hr the next 4 days; 3 days without drug; 5 days of 0.5 gm each 6 hr; 3 days off drug; and a final 5 days of 0.5 gm every 6 hr. The 10 men responded well initially and remained well during the total treatment period. Eight volunteers remained completely free of illness following cessation of treatment. Two men experienced transient, mild illness which resolved without additional treatment. One of these had low grade fever (to 100.4 F) without symptoms the 5th and 6th day after the end of the treatment. The 2nd subject had mild symptoms and slight fever (maximum of 101 F) from 3-9 days after the last drug. Thus, the interrupted schedule of tetracycline treatment begun early in the course of typhoidal tularemia, though imperfect, prevented major symptomatic relapse in 10 subjects.

To assess the importance of the various elements of the interrupted regimen, 2 additional schedules, considered as controls, are currently under study. One consists of 1 gm tetracycline every 6 hr the first day of treatment then 0.5 gm every 6 hr for 14 more days; this schedule includes the same total quantity of drug as the interrupted program but administered continuously over

a shorter time. The other is the same except that the treatment period is for a total of 21 days, the same overall duration as the interrupted regimen but given continuously and involving more drug.

These results will be of importance in the overall evaluation of an interrupted treatment schedule for treating typhoidal tularemia with broad spectrum antibiotics without relapse.

A variant of the primary interrupted schedule of tetracycline, vide supra, was evaluated in the second study. The concept under test was that 5-day courses of drug (each as in the primary regimen) would be repeated as often as necessary (not exceeding 3 courses) to keep men fully functional. The 8 subjects inhaled 23,000 (range 17,600 to 28,600) viable cells of P. tularensis, SCHU-S4. Treatment was first instituted shortly after clinical illness appeared. The men were evaluated by one physician of the Division once daily and oftener as necessary employing only such facilities as would be available in an aid station (the Ward Officer monitored the study employing all available facilities). The "aid-station" physician had had some casual experience with tularemia while in the unit but had never previously managed cases. He was told to re-treat individuals as soon as he thought it desirable in order to keep them in duty status. All 8 volunteers required a second 5-day course of tetracycline, instituted the 4th day off drug in 2 and the 5th day off drug in 6. Despite the best intentions of the "aid-station" physician the onset of relapse was frequently so rapid that he was unable to anticipate overt illness sufficiently to prevent disability. A third 5-day course of tetracycline was required in 6 subjects because of a 2nd relapse, 3 on the 4th and 3 on the 5th day off drug. The other 2 volunteers experienced mild, non-disabling relapses at the same time but recovered without drug. No further relapses were observed, however, in the men. Thus, the attempt to conserve drug by employing multiple short courses of tetracycline, as in the primary interrupted program, but administering them in anticipation of relapse rather than on schedule was noticeably ineffective. The "anticipation" was insufficiently accurate under better than average circumstances to maintain good health. Moreover, no significant saving in drug was affected--256 gm for 8 men with the primary program vs 236 gm for the variation under discussion. This regimen was complicated by the development of photoallergic eruption in 4 of the 8 volunteers, presumably related to tetracycline. Such a high incidence in one group was truly remarkable as a similar number of subjects on the primary interrupted regimen at the same time, as well as our total previous experience, had not resulted in a single such case. The particular timing of repeated short courses of tetracycline employed here is unusual in therapeusis and it might be suggested that it is an optimal one for induction of a rare complication of tetracycline administration, photoallergy.

Summary and Conclusions:

Interrupted and continuous courses of tetracycline have been evaluated in the treatment and postexposure prophylaxis of experimental respiratory

tularemia in volunteers. For treatment an interrupted course of tetracycline was effective in achieving and maintaining well-being without later major symptomatic relapse. This regimen of 4.0 gm in divided doses on day 1 followed by 4 days of 0.5 gm each 6 hr; 3 days without drug; 5 days of 0.5 gm each 6 hr; another 3 days off drug; and finally 0.5 gm at 6-hr intervals for 5 additional days. Variations of this schedule and continuous treatment for 10 days were noticeably deficient either because of illness between courses of treatment or by relapses.

Continuous administration of tetracycline, 0.5 gm twice daily, for 28 and for 42 days prevented later onset of tularemia, but 20% of subjects were more or less disabled for a short time by symptoms, possibly related to tularemia, during the course of tetracycline. Intermittent tetracycline, 0.5 gm twice on every other day over a 19 day period, failed to prevent subsequent development of overt illness.

An unusual incidence, 4 of 8 subjects, of photoallergy presumably related to tetracycline followed one particular interrupted schedule of administration.

Publications:

None.

ABSTRACT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-02:	Prevention and Treatment of Biological Warfare Casualties
Study No. -00-03:	Chemoprophylaxis and Therapy of Infectious Diseases of Potential Biological Warfare Significance
	Section III. Antibiotic Prophylaxis for Tetanus.
Reporting Installation:	U. S. Army Medical Unit Fort Detrick, Maryland
Division:	Medical
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Authors:	Robert E. Nopar, Captain, MC William D. Sawyer, Major, MC ~~Sheldon Sidell, Captain, MC~~ ~~Ralph W. Kuehne~~ Dan Crozier, Colonel, MC
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

Adult female guinea pigs were infected with a known concentration of tetanus spores. When an overwhelming dose of 50,000-100,000 spores were used, oxytetracycline even in large doses failed to show a protective effect.

With a dose of 10,000 spores, a definite protective effect was obtained manifested by delay in onset of symptoms, development of local tetanus only, and complete recovery of all animals.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-02: Prevention and Treatment of Biological Warfare Casualties

Study No. -00-03: Chemoprophylaxis and Therapy of Infectious Diseases of Potential Biological Warfare Significance

Section III. Antibiotic Prophylaxis for Tetanus.

Description:

To asesss the effect of selected antimicrobial drugs and of various drug regimens for prophylaxis and therapy of infectious diseases.

Section III. To study the efficacy of antibiotic administration for prophylaxis of tetanus in unimmunized individuals.

Progress:

Active immunization with tetanus toxoid provides effective protection from tetanus. For unimmunized individuals who incur a wound, prophylaxis is dependent upon passive protection through the administration of equine or bovine tetanus antitoxin. Because of the high incidence of serious side effects associated with the use of antitoxin, a superior method of prophylaxis is needed.

Oxytetracycline has been shown to inhibit the growth of Clostridium tetani in vitro and in vivo. Its use as tetanus prophylaxis is the subject of these studies. Previous studies at this installation (USAMU Annual Progress Report, 1963) using a meat base suspension of tetanus spores have shown that oxytetracycline has a protective effect in rabbits. In this study, a closer approximation of the usual clinical situation was achieved by the use of a saline suspension of known concentration of tetanus spores.

Tetanus organisms were inoculated into tubes of Bacto cooked meat media and incubated at 37 C for 72 hr. The supernatant fluid from all tubes was combined and centrifuged for 30 minutes at 2000 RPM. The sediment was resuspended in sterile saline, and heated at 65 C for 30 minutes to kill vegetative forms and inactivate any toxin present. Serial dilution studies using Brewer's anaerobic agar plates revealed a concentration of 10^6 spores/ml of suspension.

Tetanus was induced in adult female guinea pigs by intramuscular (IM) inoculation into the right hind leg with Cl. tetani spores in a volume of 0.1 ml mixed with an equal volume of a 5% $CaCl_2$ solution. The $CaCl_2$ aided

the germination and multiplication of the organisms by producing slight tissue damage at the injection site. Injection of $CaCl_2$ solution alone produced no apparent local or systemic effects.

The guinea pigs were divided into groups based on the number of tetanus spores and the dose of oxytetracycline. Treatment was given IM into the left hind leg in one daily dose beginning immediately after injection of the tetanus spores. A total of 3 or 5 injections at 24-hr intervals was given. Survivors were observed for 31 days.

The course of the disease and symptomatology were fairly constant with the first symptoms appearing about 48 hr postinoculation. The first sign of the disease was stiffening of the inoculated leg which remained extended and dragged behind the animal. As the disease progressed, both hind quarters became paralyzed, the animal evidenced hyperirritability, developed opisthotonus with clonic spasms, and died 1-3 days after onset of symptoms.

The results of treatment are shown in Table I. Oxytetracycline even in large doses failed to provide protection against an overwhelming inoculum (50,000-100,000) of tetanus spores. When a dose of 10,000 spores was used, onset of symptoms was delayed (3-6 days) in all animals. These animals developed mild to moderate local tetanus (stiff leg) but the disease did not become generalized. Complete recovery occurred in 1-4 weeks.

A study is in progress to see if the development of local tetanus can be prevented by a twice daily treatment regimen resulting in a more constant blood level of oxytetracycline. Further studies are planned to determine how long after infection treatment can be started and still be protective.

Summary and Conclusions:

Adult female guinea pigs were infected with a known concentration of tetanus spores. When an overwhelming dose of 50,000-100,000 spores was used, oxytetracycline even in large doses failed to show a protective effect.

With a dose of 10,000 spores, a definite protective effect was obtained manifested by delay in onset of symptoms, development of local tetanus only, and complete recovery of all animals.

Publications:

None.

TABLE I. EFFECT OF OXYTETRACYCLINE PROPHYLAXIS ON EXPERIMENTAL TETANUS IN FEMALE GUINEA PIGS

CHALLENGE *Cl. Tetani* Spores	OXYTETRACYCLINE PROPHYLAXIS mg/kg for 3 days	ONSET OF TETANUS no./total <48 hr	>48 hr	SURVIVORS
	None	6/6		0
	20	6/6		0
100,000	20 (for 5 days)	6/6		0
	30	12/12		0
	60	2/2		0
50,000	30	2/2		0
	60	2/2		0
10,000	None	5/6	1/6	42
	30		6/6	6

ANNUAL PROGRESS REPORT

Project No. 1C622401A096:	Medical Defense Aspect of Biological Warfare (U)
Task No. 1C622401A096-02:	Prevention and Treatment of Biological Warfare Casualties
Study No. -00-04:	Studies in Combined Antigens
Reporting Installation:	U. S. Army Medical Unit Fort Detrick, Maryland
Division:	Bacteriology
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Authors:	Jerome H. Greenberg, Lt Colonel, MC William C. Day, 1st Lt., VC James W. Higbee, 1st Lt., MSC
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-02:	Prevention and Treatment of Biological Warfare Casualties
Study No. -00-04:	Studies in Combined Antigens
Reporting Installation:	U. S. Army Medical Unit Fort Detrick, Maryland
Division:	Bacteriology
Period Covered by Report:	1 July 1963 to 30 June 1964
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Living vaccine strain (LVS) of Pasteurella tularensis, anthrax protective antigen (APA), tetanus-diphtheria (TD) toxoids, and smallpox (SMP) vaccine were used alone and in combination to immunize guinea pigs.

The addition of LVS to APA in a single subcutaneous injection was found to enhance markedly the protective effect of APA against anthrax without seriously interfering with the protective action of the LVS against tularemia.

Neither TD nor SMP interfered with the ability of APA to protect against subsequent anthrax challenge. However, both the TD and the SMP reduced the protective effect of LVS against tularemia challenge.

The enhancement of the protection against anthrax by APA was found to be greater when the LVS and APA were mixed prior to administration than when they were administered simultaneously but at separate sites.

LVS and APA administered percutaneously protected against tularemia but not against anthrax. However, when a subcutaneous injection of APA alone was given one month after the percutaneous LVS-APA vaccination, markedly enhanced protection against subsequent subcutaneous anthrax challenge was afforded.

Diptheria and tetanus antitoxin levels in the sera of guinea pigs given one injection of various combinations of TD, LVS, and APA were found to be increased over the levels achieved with TD alone. The increase was 10-fold for diphtheria but was less marked and less consistent for tetanus.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-02: Prevention and Treatment of Biological Warfare Casualties

Study No. -00-04: Studies in Combined Antigens

Description:

To determine the feasibility of combining various antigens and to establish the best means by which to effect and use such combinations.

Progress:

The combining of antigens for the purpose of reducing the number of required injections began in the early years of this century with work on typhoid and paratyphoid A and B vaccines, notably by such workers as Widal and Castellani (Brit Med J 2:356, 1917). TAB, as the combined vaccine came to be called, was virtually a routine immunizing agent among the armies of World War I. During the decade after the war Ramon and Zoeller (Internat Clin 1 (NS): 2, 1939) reported that TAB enhanced the immunologic response to both tetanus and diphtheria toxoids when administered in combination with these materials. This discovery added a new and cogent reason with combining antigens.

Since the original work of Ramon and Zoeller, further studies by them and by others have resulted in the routine use of such combinations as TAB, tetanus and diphtheria toxoids; diphtheria-tetanus toxoids, pertussis, and polio; smallpox and yellow fever (Dakar); and influenza and adenovirus.

In recent years the Russians have been most active in the field of combining antigens, using a wide range of materials, including living organisms, and generally reporting success. The most ambitious Russian combination reported to date has been the polyvalent vaccine of the Scientific Research Testing and Medical Institute (NIISI) containing TAB, Vibrio cholerae, Shigella dysenteriae, Shigella flexneri, Clostridium tetani. Clostridium botulinum A, B, and E, Clostridium novyi, and Clostridium perfringens. This material has been stated by Aleksandrov and Gefen (Active Specific Prophylaxis of Infectious Diseases and Its Improvement, Moscow, 1962) to be highly effective in humans except for the Cl. perfringens component.

The Russian workers Saltykov and Zemskov (Zh Mikrobiol (Engl) 31:653, 1960) have combined Cl. botulinum A and B, Cl. perfringens, and Cl. novyi with living Pasteurella pestis and Pasteurella tularensis and used it with reported success in animals. This combination of living organisms with chemical vaccines or toxoids is the only such combination known to us prior to the work reported here.

Of the 4 materials used in the present studies, tetanus and diphtheria toxoids in combination and smallpox vaccine have been standard, routine immunizations. Living vaccine strain of P. tularensis has been a special purpose vaccine, and is generally accepted as effective both in the United States and Russia. Anthrax protective antigen, also a special purpose vaccine, has not been studied in humans to the same extent as has LVS, although evidence from U. S. woolen mills suggest that it has some protective effect. The Russian anthrax preparation is a living spore suspension from an attenuated strain of Bacillus anthracis, and it is one of the few materials which the Russians themselves have admitted to be relatively ineffective.

Animals: Hartley strain guinea pigs of 400 gm, individually identified, were used in all experiments.

Vaccines and Immunization Procedures: The vaccines used singly or in combination were as follows:

1. Anthrax protective antigen (APA) was commercially produced material (J Bact 85:230, 1963). It was made available to us through the kindness of Dr. G. G. Wright, USABL. In some instances the APA was washed and resuspended in order to remove the 1:40,000 benzethonium chloride preservative.

2. The tularemia vaccine was living attenuated strain (LVS) (J Infect Dis 91:86, 1952) produced by USABL and the National Drug Co.

3. The tetanus-diphtheria (TD) toxoids were the standard combined materials for adult (National Drug Co.).

4. The smallpox (SMP) vaccine was the standard freeze-dried material (Wyeth Laboratories).

Immunization was by either the subcutaneous (SC) or percutaneous (PC) routes. With the former, the dose varied from 0.5-1.0 ml while with the latter, the multiple pressure (30 times) method was used.

In Experiment No. 3 one group of animals was given SC LVS and APA simultaneously, but at separate sites. Several groups of PC-immunized animals were given a second injection of APA subcutaneously 2 months after the original immunization.

The total dose of APA and LVS given SC varied in the 3 experiments as follows: in Experiment No. 1 the 0.6-ml APA dose injected was equal to 0.5 ml of the original vaccine and the injected dose of LVS was approximately 3×10^5 organisms. In Experiment No. 2 the APA dose of 0.5 ml was equal to 0.5 ml of the undiluted vaccine and the injected dose of LVS was approximately 3×10^5 organisms. In Experiment No. 3 the subcutaneous APA dose of 0.50 ml was equal to 0.25 ml of undiluted vaccine and the injected dose of LVS was approximately 3×10^5 organisms.

For PC vaccination, the vaccines were adjusted so that the APA contained therein equalled 0.17 ml of undiluted vaccine and the LVS concentration was approximately 3 x 10^6 organisms/ml. The second dose of APA, given SC, was 0.5 ml of undiluted vaccin..

The TD toxoids as administered contained 0.4 ml of the original undiluted material, and the SMP vaccine was adjusted to 1/3 of its original concentration.

Challenges: Appropriately selected groups of vaccinated and control animals were challenged by SC injection with 1 x 10^3 organisms of the virulent SCHU-S4 strain of F. tularensis 2 weeks after immunization. Other groups were challenged by SC injection with 1 x 10^3 spores of either Vlb-189 or NH-6 strains of B. anthracis at 4 weeks after immunization.

Antibody Determinations: Sera were drawn prior to immunization and challenge and also from survivors before sacrifice. Examinations for tularemia antibody and for antibody to APA were performed in this laboratory by the standard tube agglutination test and the gel diffusion test (J Gen Microbiol 17:505, 1957).

Diphtheria and tetanus antitoxin levels were determined by the Massachusetts State Institution of Laboratories and arrangements for smallpox antibody determinations are being made with the University of Colorado School of Medicine.

Bacteriologic Examinations: Blood smears and cultures were done at death or sacrifice on all animals challenged with anthrax. In addition, cultures of spleen emulsions were done on all animals dying 2 or more weeks after challenge, and on all sacrificed animals.

Blood, liver, and spleen cultures on appropriate media from all animals challenged with F. tularensis were examined for typical colonies which were then confirmed by the slide agglutination test.

The results of the anthrax challenge of all of the animals immunized by the subcutaneous route are summarized in Table I.

Several points of interest are apparent from the table. The most striking is the marked superiority of the APA-LVS combination to APA alone in protecting guinea pigs against challenge with both B. anthracis strains Vlb-189 and NH-6. The combination protected 34/34 animals against Vlb-189 in 3 replicates and 22/31 animals against NH-6, also in 3 replicates.

The single experiment in which the APA and LVS were injected simultaneously but in separate sites seems to indicate that the antigens must be physically mixed in order to obtain enhanced protection against NH-6.

TABLE I. RESPONSE OF GUINEA PIGS GIVEN SINGLE SC DOSE OF VARIOUS VACCINES TO CHALLENGE WITH 2 STRAINS OF B. ANTHRACIS, 10^3 SPORES

VACCINE[a]	Vlb-189								NH-6			
	17-DAY SURVIVORS		MEAN DAY OF DEATH		17-DAY SURVIVORS		MEAN DAY OF DEATH					
	Total	Experiment			Total	Experiment						
APA	13/32	1/11[b]	8.0		1/11		8.0					
		5/11	5.5									
		5/10	8.8									
APA-LVS	34/34	14/14[b]			22/31				6/11		7.0	
		9/9							6/11[b]		12.0	
		11/11[b]							6/10		7.8	
APA-LVS (Separate sites)					3/12						3.3	
APA-TD	7/12		4.8									
APA-LVS-TD	6/11		5.9									
Controls	0/26	0/14	1.7		0/21				0/10		2.8	
		0/12	2.4						0/11		3.3	

a. APA: anthrax protective antigen.
LVS: living vaccine strain of F. tularensis.
TD: tetanus-diptheria toxoids.

b. APA washed and resuspended.

Repeated experiments with small groups of animals by several workers have indicated that the survival of guinea pigs when immunized with APA alone and challenged with Vlb-189 at 4 weeks postimmunization averages about 50% with a range of 30-70%. Therefore, no significance can be attached at this time to the single experiments in which the combinations of APA-TD and APA-LVS-TD afforded slightly better protection than did APA alone.

It is apparent from Table II that APA given PC, whether alone or in combination, gave no protection whatsoever against Vlb-189 challenge. The 4 groups of animals given a second dose of APA by SC injection were unchallenged vaccine controls which were reimmunized as a pilot study.

TABLE II. RESPONSE OF GUINEA PIGS VACCINATED ON TWO SCHEDULES AND CHALLENGED SC WITH 10^3 SPORES OF B. ANTHRACIS, V19-189 ONE MONTH AFTER LAST VACCINE ADMINISTRATION

VACCINES[a] APA	LVS	SMP	CHALLENGED AT 1 MONTH LD[illegible]	MEAN DAY OF DEATH
PART A. Single SC dose of vaccine.				
-	+	-	8/10	2.6
+	-	-	8/12	2.7
+	+	-	8/11	2.7
+	-	+	8/11	2.4
+	+	+	8/12	2.4
-	-	-	8/10	2.3
PART B. Same as A, with single SC booster injection of APA at 2 months; challenged 3 months.				
+	+	-	12/12	
+	+	+	8/12	3.5
+	-	+	7/12	3.6
-	+	+	7/11	3.3
-	-	-	8/10	3.2

a. APA: anthrax protective antigen.
LVS: living vaccine strain of F. tularensis.
SMP: smallpox vaccine.

Although the necessary controls were not included in this pilot study, it is apparent from the results that the percutaneous APA-LVS followed by subcutaneous APA gave better protection than the subcutaneous APA alone would have done. The protection in the other 3 groups is of the magnitude of APA alone

at its best. The possibility that the prior percutaneous immunization improved the protection somewhat in these groups cannot be ruled out. Further study is necessary to determine if such was the case.

Table III summarizes the results of tularemia challenge in guinea pigs immunized SC and PC. The APA-LVS combination clearly provided protection which was equal to or better than that of LVS alone. Other

TABLE III. RESPONSE OF VACCINATED GUINEA PIGS TO SC CHALLENGE WITH 10^3 F. TULARENSIS SCHU-S4 ORGANISMS

VACCINE	SC EXPOSURE		MEAN DAY OF DEATH
	Total	Experiment	
Subcutaneous			
LVS	1/26	0/14	14.3
		1/12	11.0
LVS-APA	3/25	3/14	14.1
		0/11	12.0
LVS-TD	0/12		14.3
LVS-TD-APA	0/11		9.2
Controls	0/26	0/15	4.0
		0/11	8.1
Intraperitoneal			
LVS	3/12		13.9
LVS-APA	5/11		13.2
LVS-SMP	0/12		14.0
LVS-APA-SMP	0/11		12.0
Controls	0/9		7.4

a. LVS: living attenuated strain of F. tularensis.
APA: anthrax protective antigen.
TD: tetanus-diphtheria toxoids.
SMP: smallpox vaccine.

combinations, with one exception, [illegible] as good as that of LVS or [illegible], [illegible]. The one exception was the LVS-[illegible]-APA [illegible]. The results of serologic testing are summarized in Tables IV, V and VI.

TABLE IV. [illegible] OF VACCINATED [illegible] B. ANTHRACIS V1B-1[illegible] AS RELATED TO [illegible] ANTIBODY TITER

VACCINE[a]	RECIPROCAL ANTIGEN TITER[b]	SURVIVORS/TOTAL	[illegible]
APA	2	0/1	[illegible]
	4	-	
	8	1/3	[illegible]
	16	2/3	[illegible]
	32	0/4	
LVS-APA	8	2/4	
	16	0/7	
	32	-	
	64	1/1	
Controls	< 2	0/[illegible]	
	2	0/1	

a. LVS: living vaccine strain of P. tularensis. APA: anthrax protective antigen.

b. All preimmunization titers were < 2.

As has been noted by other workers, [illegible] not correlate with survival of individuals after [illegible] little, if any, correlation between prechallenge [illegible] survival after challenge.

It is apparent from Table VI that LVS and/or APA, [illegible] increased diphtheria antitoxin levels 10-fold by the [illegible]. Although tetanus antitoxin levels were not elevated similarly by the combinations of antigens, the fact that there was little or no [illegible] on the response is of importance.

The mechanism of the adjuvant action of LVS on APA [illegible]. Similar effects on other antigens by the endotoxins of various [illegible] have been reported. In these instances however, the living [illegible] used as was the case in this study. Consequently the mechanism involved may have been completely different.

a.

b. Reading levels at day 3 were < 0.001 units/ml for diphtheria and < 0.005 units/ml for tetanus.

Among the possible ways in which the adjuvant [illegible] be [illegible] are: absorption of the APA to the LVS with [illegible] more widespread distribution; local irritation with [illegible] effect; stimulation of antibody-producing tissue by LVS [illegible] plastic providing more [illegible] for immunologic [illegible] lation of phagocytic activity resulting in [illegible] the APA in such a way as to render it more [illegible] that the enhancement of the protection against [illegible] phenomenon, but is instead an increased resistance resulting [illegible] alterations within the host.

Farthing and Cole (J [illegible] 60:411, 1953) [illegible] adjuvant action of killed [illegible] the result of either [illegible] or [illegible] reported an adjuvant effect of [illegible] or B. [illegible] [illegible] They [illegible] cells in the [illegible] and lymph nodes, and that [illegible] same when the [illegible] given in separate [illegible].

[illegible] et al. [illegible] with [illegible] action [illegible] distribution of [illegible] in the [illegible] permeability, or from modified phagocytic [illegible].

The apparent [illegible] of an [illegible] antigen by [illegible] of APA [illegible] there has been no previous report of such an [illegible] by a [illegible] administered SC.

The studies to date indicate not only that it may be [illegible] to combine the antigens involved, but that an important and [illegible] enhancement of [illegible] protection may be [illegible].

Summary and Conclusions:

The results of a study of the antigenic effectiveness of various combinations of living vaccine strain of P. [illegible], anthrax protective antigen, tetanus and diphtheria toxoids, and [illegible] are reported. The materials were used to immunize guinea pigs by the SC and [illegible] routes and efficacy was tested by challenge and/or serologic tests.

The protection achieved by APA against V1b-129 and NH-6 strains of B. anthracis was markedly enhanced by combining the APA with LVS prior to SC administration. Similar enhancement was demonstrated by PC administration of the APA-LVS combination when followed in 2 months by a SC injection of APA alone.

Protection against tularemia challenge was elicited with ZMA alone or in combination with ATH when administered by single sub cutaneous injection. The protection afforded by other combinations may also be significant.

Diphtheria antitoxin levels were consistently higher when ZMA was administered in combination with other antigens, however, the antitoxin levels after administration of the combinations were found to be lower than the levels achieved by ZMA alone.

Publications:

None.

ANNUAL PROGRESS REPORT

Project No. 1C[illegible]: Medical Defense Aspects of Biological Agents (U)

Task No. 1C[illegible]: Prevention and Treatment of Biological Warfare Casualties

Study No. [illegible]: Development and Evaluation of an Effective Vaccine Against Pneumonic Plague

Reporting Installation: U.S. Army Medical Unit [illegible]

Division: [illegible]

Period Covered by Report: [illegible]

Professional Author(s): [illegible]

Reports Control Symbol: [illegible]

Security Classification: [illegible]

ABSTRACT

Project No. 1C622401A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1C622401A096-02: Prevention and Treatment of Biological Warfare Casualties

Study No. [illegible]-12-01: Development and Evaluation of an Effective Vaccine Against Pneumonic Plague

Reporting Installation: U.S. Army Medical Unit, Forest Glen Section, Washington, D.C.

Division: Microbiology

Period Covered by Report: 1 July 1963 to 30 June 1964

Professional Authors: John D. Marshall, Jr., Major, MSC
[illegible], 1st Lieutenant, MSC
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Reports Control Symbol: RCS-MEDDH-288

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Six antigens have been partially purified from Pasteurella pestis, strain EV 76, and 2 from [illegible] tuberculosis, strain [illegible]. [illegible] results of [illegible], each antigen produced a single heavy precipitin line when tested against a hyperimmune rabbit anti P. pestis antiserum. Antisera prepared against each antigen showed one heavy precipitin line when tested against a crude extract of acetone killed and dried P. pestis EV 76 cells. One of the antigens has been identified as the lipopolysaccharide antigen of Davies. The remaining 7 antigens did not react in any of the 11 known P. pestis antigen antibody reactions. Protection tests utilizing 0.01 mg to 1.0 mg of each antigen per mouse were negative. Further studies are in progress to characterize the 8 isolated antigens and to isolate additional antigens.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1C622401A096-02: Prevention and Treatment of Biological Warfare Casualties

Study No. -12-01: Development and Evaluation of an Effective Vaccine Against Pneumonic Plague

Description:

To determine the specific antigens conferring a relative immunity against pneumonic plague and the most appropriate route of immunization and to develop methods of assessing the host's response to such immunization.

Progress:

An effort was initiated some 15 months ago to isolate as many antigens as possible from [illegible] pestis in order to test their ability to serve as immunizing agents against challenge with P. pestis. Studies [illegible] by the U.S. Army Biological Laboratories, Fort Detrick, Md. (J [illegible] 84:478, 1962) indicated that P. pestis contains at least 18 [illegible] antigens as [illegible] double diffusion tests. Many of these antigens have been studied in detail by various laboratories. The F antigen [illegible] has [illegible] attention. [illegible] and [illegible], and have shown it to be an effective immunizing agent against [illegible] plague. The V and W antigens have been studied by both the [illegible] and [illegible] groups [illegible] Path 37:481, 1956). The V factor has been shown to be an effective immunizing antigen, while the W factor does not appear to elicit any degree of protective immunity in experimental animals. The T antigen (murine toxin) was purified and characterized by Ajl and his group (J Bact 70:158, 1955). This antigen, while [illegible] plague infection, did not significantly alter the mortality rate. [illegible] and [illegible] have shown that P. pestis strains devoid of [illegible] F, V, and W, when used as vaccines, protected animals against challenge infection with a fully virulent strain. They concluded that there are one or more yet unidentified protective antigens in P. pestis. The work initiated in this laboratory is directed toward the isolation, purification and characterization of the remaining antigens.

An avirulent strain of P. pestis (EV-76) was found to be a source of at least 12 antigens other than the ones mentioned above. Extracts of an acetone powder prepared from this strain showed at least 14 separate and distinct precipitin lines in an Ouchterlony double diffusion test employing

polyvalent antisera. $(NH_4)_2SO_4$ fractional precipitation was chosen as the method of treatment which would separate the acetone powder extract into groups of antigens. Various $(NH_4)_2SO_4$ "cuts" were prepared; each shows a characteristic Ouchterlony double diffusion pattern. Disc electrophoresis was employed as analytical tool to estimate the minimum number to proteins in each fraction. Table I shows the $(NH_4)_2SO_4$ "cuts" prepared, the number of Ouchterlony lines present in each, and the minimum number of proteins appearing on electrophoretograms. Although all the $(NH_4)_2SO_4$ fractions are cross contaminated with various antigens from adjacent cuts, each one appears to contain a maximum concentration of at least one antigen (possible exception of 0-27% fraction).

TABLE I. NUMBER OF COMPONENTS OBTAINED BY VARIOUS IDENTIFICATION TECHNIQUES FROM $(NH_4)_2SO_4$ PRECIPITATES

% SATURATION	DOUBLE DIFFUSION	[illegible]	[illegible]
0-27	-	3	3
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
Supernatant	[illegible]	[illegible]	[illegible]

[illegible] been made to devise a further purification step for each of these fractions in order to partially purify one or more antigens from each fraction. Partially purified proteins are designated as antigens when sufficiently pure to (1) show a single heavy precipitin line in an Ouchterlony test when reacted against a hyperimmune antiserum and (2) show a single heavy precipitin line when reacted against homologous antiserum. To date, procedures have been developed for 4 of the 8 $(NH_4)_2SO_4$ cuts and 6 antigens have been partially purified from the 4 cuts in amounts varying from 20 to 800 mg. The 0-27% cut can be fractionated by DEAE-cellulose ion-exchange chromatography to yield 2 antigens (only one of which has been studied [illegible]). Sephadex gel filtration after heat treatment yields an antigen from the 47-63% cut which has been identified as the

lipopolysaccharide (LPS) antigen of Davies (Biochem J 63:105, 1956). Davies' preparation is a protein-free lipopolysaccharide hapten which was antigenic only after conjugation to a protein preparation whereas the present antigen is about one-half protein and is antigenic for rabbits. Hanging curtain electrophoresis (Beckman Model CP) of the 70-100% cut, after protamine sulphate treatment to remove nucleic acid, yields preparations of the LPS antigen and a new antigen. Finally, ultrafiltration of the $(NH_4)_2SO_4$ supernatant yields an antigen of rather small size. Antisera to this new antigen has been prepared successfully in only a single animal and this preparation was not stable. Although polyvalent antisera shows a positive precipitin line with this antigen, methods for monovalent sera production are being studied.

An additional antigen was extracted from the acetone powder by 1 M potassium thiocyanate. Further purification by heat treatment and Sephadex gel filtration yields an antigen of large molecular weight.

In addition, two antigens have been partially purified from Pasteurella pseudotuberculosis strain 32 employing $(NH_4)_2SO_4$ fractional precipitation and DEAE-cellulose chromatography. These two additional antigens appear to be different from any previously discussed and are also found in P. pestis.

Antisera against the previously studied Fraction 1 and toxin do not react with any of the antigens discussed here. Comparison with Fort Detrick standards (by Dr. William Lawton) shows the 7 isolated antigens to be different from their 11 standards (antigens D, E, F, I, K, L, LPS, Q, T, V, W).

Six antigens were used to immunize mice against challenge with virulent P. pestis (Table II). Results indicate that these antigens are nonprotective against a challenge dose of approximately 1,000 LD_{50}. An attempt will be made to study the number and amount of contaminants in each preparation, the approximate molecular weight and isoelectric point of each antigen and the content of carbohydrate and lipid in each.

TABLE II. SURVIVAL OF IMMUNIZED[a] MICE CHALLENGED[b] WITH 1,000 LD_{50} *P. pestis* STRAIN 195P

TOTAL PROTEIN mg	SURVIVAL OF MICE (No. Survivors/No. Challenged) USING ANTIGEN					
	LPS (Davies)	1	3	4	5	Fraction 1[c]
1.0	0/10	0/5	0/9	0/10	3/10	
0.1	1/20	0/10	0/9	0/10	1/10	
0.01	0/10	0/10	0/10	0/10	0/10	
0.001	1/20	0/10	0/10	0/10	0/10	
0.0001	1/20	0/9	0/10	0/10	0/10	
0.25						10/10
0.025						10/10
0.0025						3/10

a. 2 equal doses with adjuvant 1 week apart.

b. 3 weeks after immunization.

c. Separate experiment.

Summary and Conclusions:

Six antigens have been partially purified from an acetone powder of *P. pestis* and 2 antigens have been partially purified from *P. pseudotuberculosis*. All but one (LPS) of these antigens have not been previously characterized. Protection tests and physical characteristics of each are being investigated.

Publications:

None.

ANNUAL PROGRESS REPORT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-02:	Prevention and Treatment of Biological Warfare Casualties
Study No. -20-01:	Efficacy of Vaccination in Anthrax Infections
Reporting Installation:	U. S. Army Medical Unit Fort Detrick, Maryland
Division:	Bacteriology
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Authors:	Martha K. Ward, Captain, USPHS Margaret L. Huff, M.P.H.
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-02:	Prevention and Treatment of Biological Warfare Casualties
Study No. -20-01:	Efficacy of Vaccination in Anthrax Infections
Reporting Installation:	U. S. Army Medical Unit Fort Detrick, Maryland
Division:	Bacteriology
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Authors:	Martha K. Ward, Captain, USPHS Margaret L. Huff, M.P.H.
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

Work to study the role of phagocytosis in acquired resistance to anthrax infections has been initiated. Preliminary studies have included *in vivo* and *in vitro* studies using the guinea pig as test animal. Spores, noncapsulated cells, and capsulated cells have been used in one or more experiments. With the techniques employed, engulfment of spores and noncapsulated cells of both virulent and avirulent strains of *Bacillus anthracis* has been demonstrated. Engulfment of fully capsulated cells has not been observed. In future studies, attempts will be made to determine: (a) the fate of engulfed spores in cells from normal and immunized animals, and (b) whether or not there is a difference in ability of spores of avirulent and virulent strains to germinate in phagocytic cells after engulfment.

BODY OF REPORT

Project No. 1C622401A096. Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-02: Prevention and Treatment of Biological Warfare Casualties

Study No. -20-01: Efficacy of Vaccination in Anthrax Infections

Description:

1. To evaluate the efficacy of several anthrax vaccines in various species of animals challenged by parenteral and aerosol routes with several different strains of Bacillus anthracis.

2. To study the pathogenesis of anthrax infection in partially protected animals.

3. To study basic mechanisms involved in development of acquired immunity to anthrax infections.

Progress:

A considerable amount of time has been spent in reviewing and summarizing results of work reported in the last two annual reports. A manuscript, "Studies on Anthrax Infections in Immunized Guinea Pigs" has been submitted for publication.

Additional work on several experimental vaccines for immunization of guinea pigs is included in the section of this report dealing with combined vaccines (See Study -02-00-04).

It has not been feasible during this report period to extend the preliminary work in monkeys reported last year.

The basic mechanisms involved in the production of acquired immunity to anthrax infections are still unknown. The difference in response of guinea pigs immunized with protective antigen to challenge with different strains of anthrax and the marked enhancement of protection against both types of strains in those animals given anthrax protective antigen combined with the living tularemia vaccine raise interesting questions of considerable significance. In addition, the necessity for more basic information on the mechanisms involved in protection against anthrax infection has been emphasized by these studies.

It is well established that phagocytosis may be of decisive importance in resistance to a number of bacterial infections. It has been suggested by

several authors that this phenomenon may be of importance in resistance to anthrax infections. Definitive evidence is, however, lacking. For example, it has never been conclusively demonstrated whether or not either circulating leukocytes or fixed macrophages are capable of engulfing fully capsulated vegetative cells in which form the body has to deal with in greatest numbers, and most probably, within a very short time after exposure. In addition, nothing is known, so far as we are aware, about the fate of spores which are readily engulfed by phagocytic cells. It has indeed been suggested that spores are disseminated in the host by this means.

During the past few months studies have been initiated with the purpose of examining the possible role of phagocytosis in protection against anthrax infections in the guinea pig. Most of the effort of the one individual working full time on this problem has been spent in attempts to develop satisfactory in vivo and in vitro procedures, and in working out technical problems encountered. Three strains of anthrax have been used in one or more experiments: the avirulent Weybridge strain and the virulent V1b-189 and NH-6 strains. Progress of phagocytosis under several experimental conditions has been followed by the smear technique at varying time intervals and in some instances with phase microscopy. The in vivo system used has been that of intraperitoneal (IP) inoculation of guinea pigs with serial sacrifice and harvest of exudate. In vitro systems have included previously described techniques and a variety of modifications.

The maintenance of guinea pig polymorphonuclear leukocytes in vitro for period of time up to 24 hr, either uninfected or after they had engulfed spores of Bacillus anthracis has presented the most difficulty. The possibility of using monocytes and/or lung macrophages for these studies is being investigated.

This work is still in a very preliminary phase and all techniques have not yet been worked out or standardized. Results of several types of exploratory experiments however, may be summarized as follows:

1. Spores of both avirulent and virulent strains are readily engulfed under all conditions examined both in vivo and in vitro.

2. Young, noncapsulated vegetative cells (chains of 1-4 cells) are also actively engulfed by guinea pig leukocytes.

3. Under the same experimental conditions, including two attempts with the surface phagocytosis techniques of Wood and co-workers (J Exp Med 83-84:397, 1946), engulfment of young fully capsulated cells could not be demonstrated.

4. When preparations of leukocytes and capsulated cells were observed with phase microscopy, leukocytes appeared, in general, to be

repelled by the capsulated organisms and the one or two cells attempting to engulf such cells failed to do so. Since there were some ungerminated spores in the preparations, and some of these were observed to be engulfed by the same leukocytes failing to phagocytize capsulated vegetative cells, it would appear that phagocytic capability of the leukocyte preparation per se was not depressed by the presence of the capsulated cells. Further studies with surface phagocytosis techniques are in progress.

5. The results of a typical exploratory experiment employing an in vivo phagocytic system are summarized in Table I. The procedure used was as follows:

TABLE I. PHAGOCYTOSIS OF SPORES OF TWO STRAINS OF B. ANTHRACIS AFTER INTRAPERITONEAL INOCULATION IN GUINEA PIGS

HOURS AFTER INOCULATION	STRAIN (Dose- 1 x 10^9 spores)	PHAGOCYTOSIS-100 POLYS					COMMENTS
		%	No. of spores per cell				
			0	1-10	10-20	20-30	
1½	V1b-189	99	1	3	31	65	Numerous free spores. Some extracellular germination. Leukocytes contain both spores and germinated cells.
	Weybridge	100	0	3	39	58	
3	V1b-189	84	16	67	16	1	Leukocytes more numerous than at 1½ hours.
	Weybridge	95	5	81	13	1	Leukocytes more numerous than at 1½ hours. Germination of intracellular spores?
5	V1b-189	33	67	22	11	0	No free spores. No germinated cells intracellularly.
	Weybridge	42	58	27	15	0	No free spores. Germination of intracellular spores.

Three guinea pigs were inoculated IP with 1 x 10^9 spores of strain V1b-189 and 3 with a similar dose of Weybridge spores. At 1½, 3 and 5 hr after injection one of each group of animals was sacrificed and peritoneal washings collected and examined by stained smears and phase microscopy. In this particular experiment heparinized blood was collected by cardiac puncture and impression smears of spleen and liver were made on all animals at time of sacrifice.

Although the concentration of leukocytes present in exudates collected at 1½ hr after spore challenge was much less than at the 3 and 5-hr time intervals, virtually all cells had ingested one or more spores and approximately 95% of the 100 counted had taken up 10 to 30 spores each. With time, the number of

leukocytes increased and the percentage of phagocytosis decreased. This apparent drop in phagocytic activity is probably a reflection of the decrease in relative concentration of spores to leukocytes as the result of very active phagocytosis observed at 1½ hrs. There was some suggestion both in smears and by phase microscopy that Weybridge spores germinated after ingestion by leukocytes and that spores of V1b-189 did not during the time period of the experiment. This observation is open to question because of presence of some germination of extracellular spores at some of the same time periods. This is an interesting question and experiments are now being designed to determine whether or not there is a difference in intracellular germination of spores of the virulent and avirulent strains.

By 1½ hrs after IP inoculation the concentration of organisms in the circulating blood was greater than 10^3/ml in the case of both strains as evidenced by the growth in pour plates containing 1 ml of blood. At the same time period, some free spores and leukocytes containing spores were observed in impression smears of both spleen and liver. It will be of interest to repeat this study using more quantitative techniques and histological methods to follow the movement of, and to determine the fate of spores, ingested by leukocytes after inoculation by IP and other routes of challenge.

Efforts to define optimal conditioning for maintaining phagocytes after they have engulfed spores of various strains of B. anthracis so that the fate of these spores may be studied will be continued. Since polymorphonuclear cells are more fragile and difficult to maintain in vitro, experiments to determine the feasibility of using monocytes or lung macrophages are being initiated.

Summary and Conclusions:

Preliminary work designed to study the role of phagocytosis in acquired immunity to anthrax infections has been initiated using the guinea pig as the test animal. Work to date has been concerned primarily with development and standardization of appropriate techniques.

In the systems examined it has been demonstrated that spores and noncapsulated cells of both avirulent and virulent strains are readily engulfed by phagocytes (mainly polymorphonuclear cells in preparations used) of normal guinea pigs. Engulfment of fully capsulated cells of the virulent strain has not been demonstrated.

As soon as appropriate techniques are worked out and standardized, attempts will be made to determine: (1) the fate of engulfed spores in phagocytes from normal and immune guinea pigs, and (2) whether or not capsulated vegetative cells can be engulfed and destroyed by phagocytes from immune animals.

Publications:

None.

ANNUAL PROGRESS REPORT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-02:	Prevention and Treatment of Biological Warfare Casualties
Study No. -40-01:	Reduction of Reactogenicity of Attenuated Vaccine Virus
Reporting Installation:	U. S. Army Medical Unit Fort Detrick, Maryland
Division:	Virology
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Authors:	Robert W. McKinney, Major, MSC Wayne Grogan, Lt Colonel, VC Marie L. Miesse Louis F. Maire, III Helen H. Ramsburg
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-02:	Prevention and Treatment of Biological Warfare Casualties
Study No. -40-01:	Reduction of Reactogenicity of Attenuated Vaccine Virus
Reporting Installation:	U. S. Army Medical Unit Fort Detrick, Maryland
Division:	Virology
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Authors:	Robert W. McKinney, Major, MSC Wayne Grogan, Lt Colonel, VC Marie L. Miesse Louis F. Maire, III Helen H. Ramsburg
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

Results of investigations involving the freeze-drying and use of attenuated Venezuelan equine encephalomyelitis are presented.

The results indicate the virus is stable during the freeze-dry processing in that little or no loss of infectivity occurs during the cycle.

The responses of man to inoculation with the reconstituted dried product did not differ from previous experience. Approximately 95% of persons administered 7000 guinea pig median immunizing doses of virus developed significant levels of hemagglutination-inhibiting antibody.

Investigation of the stability of the freeze-dried virus is continuing as well as its use for immunizing "at risk" personnel.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-02: Prevention and Treatment of Biological Warfare Casualties

Study No. -40-01: Reduction of Reactogenicity of Attenuated Vaccine Virus

Description:

Investigations in this study are directed toward development of a live attenuated virus vaccine for immunization of man to infection with Venezuelan equine encephalomyelitis virus.

Progress:

The use of attenuated Venezuelan equine encephalomyelitis (VEE) virus for immunization of "at risk" personnel has continued. The virus suspension employed was prepared as described in the FY 1962 Annual Report with the addition of cabinet freeze-drying.

Freeze-Drying of Attenuated VEE Virus. A single production lot of attenuated virus (TC 83/3-2) was prepared and titrated in guinea pigs. Following titration the material was diluted with Hanks balanced salt solution (BSS) containing 0.5% human serum albumin (HSA) USP to a concentration of $10^{6.2}$ guinea pig intraperitoneal immunizing doses$_{50}$ ($GPIPID_{50}$)/0.5 ml. This value was calculated to provide for (a) an expected loss of approximately 0.8 logs on drying and (b) a 0.5 ml dose containing $10^{3.7}$ $GPIPID_{50}$ following reconstitution to 50 ml with Hanks BSS plus 0.5% HSA.

The diluted material was dried in 1.0 ml volumes using 5.0 ml bottles in an NRC 3501 cabinet dryer having a capacity of 230 bottles. A drying time of 22 hr was employed for each of 13 lots. Following drying the bottles were capped and hermetically sealed in metal cans containing desiccant and stored at -20 C.

Bottles were selected at random from each drying lot for sterility and potency tests. No evidence was found of bacterial, mycotic or viral contaminants.

Results of potency assays are presented in Table I. The individual lots were titrated in guinea pigs employing 6 animals per dilution.

The titers following drying are higher than anticipated except for Lots 3, 6 and 11. From these results it would appear that the estimated 0.8-log

TABLE I. TITERS OF 13 LOTS OF CABINET DRIED ATTENUATED VEE VIRUS.

LOT NO.	TITER (LOG_{10})	LOT NO.	TITER (LOG_{10})
1	6.3	8	6.6
2	6.3	9	6.4
3	5.6	10	6.8
4	5.5	11	5.5
5	6.5	12	6.5
6	5.8	13	6.3
7	6.3		

loss did not occur. Results obtained with the use of Lot 3 in man are presented later in this report.

For purposes of obtaining information relative to the stability of the dried product, Lot 13 was divided according to the schema in Table II.

TABLE II. STABILITY TEST PATTERN FOR ATTENUATED VEE VIRUS, LOT 13.

TYPE MATERIAL	TEMPERATURE °C			
	Room	+4	-20	-70
Wet, frozen			x	x
Dried	x	x	x	x
Dried, sealed w/desiccant	x	x	x	x

Samples from each of the test temperatures will be titrated at 90 days after drying. If this test gives evidence of deterioration the assays will be repeated in 30 days. The 90-day assay results are not complete at this writing.

Studies in Man. As noted above Lot 3 of TC 83/3-2 has been employed for immunization of "at risk" personnel. The dose administered has been 0.5 ml subcutaneously containing approximately 7000 $GPIPID_{50}$. This is an increase of 7-fold over the dose previously employed. Of 776 persons inoculated 12, or 1.5% failed to develop demonstrable hemagglutination-inhibiting (HI) antibody

by 28 days following inoculation. An additional 17 persons, or 2.2% showed a minimal response, i.e., 1:10. Thus, at the dose level employed significant HI antibody responses were obtained in 96.3% of those inoculated. Systemic reactions were of the same order as previously observed.

For purposes of comparison a summary of the HI responses obtained with the several different passage levels and lots of attenuated virus are presented in Table III.

TABLE III. RESPONSE TO VARIOUS PASSAGE LEVELS OR LOTS OF ATTENUATED VEE VIRUS BASED ON HEMAGGLUTINATION-INHIBITION TEST.

PASSAGE LEVEL OR LOT	NEGATIVE PREBLEED Postinoculation Titer			POSITIVE PREBLEED Change in Titer		
	≥1:20	1:10	Neg.	≥4X	2X	None
TC 50	11	0	0	1	2	4
TC 80	52	0	0	14	4	10
TC 81/2-4	31	1	3	2	5	21
TC 82/2-4	5	0	0	0	0	0
TC 82/2-9	684	21	18	295	149	320
TC 83/3-2	776	17	12	0	1	0
Total	1559	39	33	312	161	355
% of total	95.4	2.5	2.1	37.6	19.4	42.8

As may be seen the responses obtained with the dried product (TC 83/3-2) compare favorably with those of other lots.

Summary and Conclusions:

The results obtained in animals and man following inoculation of reconstituted attenuated Venezuelan equine encephalomyelitis virus indicate this agent can be freeze-dried without significant loss in infectivity. The similarity of titration results for 13 separate "drying lots" serves to emphasize the reproducibility of the drying procedure.

These studies will be continued particularly as regards stability of the dried product.

Publication:

1. McKinney, R. W., Berge, T. O., Sawyer, W. D., Tigertt, W. D., and Crozier, D.: "Use of an Attenuated Strain of Venezuelan Equine Encephalomyelitis Virus for Immunization in Man," Amer J Trop Med Hyg 12:597-603, 1963.

ANNUAL PROGRESS REPORT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-02:	Prevention and Treatment of Biological Warfare Casualties
Study No. -40-02:	Asrogenic Immunization with Attenuated Venezuelan Equine Encephalomyelitis Virus
Reporting Installation:	U.S. Army Medical Unit Fort Detrick, Maryland
Division:	Medical
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Authors:	Thomas J. Smith, Major, MC William D. Sawyer, Major, MC Ralph W. Kuehne
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-02:	Prevention and Treatment of Biological Warfare Casualties
Study No. -40-02:	Aerogenic Immunization With Attenuated Venezuelan Equine Encephalomyelitis Virus
Reporting Installation:	U.S. Army Medical Unit Fort Detrick, Maryland
Division:	Medical
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Authors:	Thomas J. Smith, Major, MC William D. Sawyer, Major, MC Ralph W. Kuehne
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

Serological follow-up of monkeys (Macaca mulatta) exposed to aerosolized attenuated Venezuelan equine encephalomyelitis virus (National Drug Company Lot-4), as described in last year's Annual Report has been completed. Presented doses ranged from 48,000-112,000 guinea pig intraperitoneal median immunizing doses. Only 4 of 8 monkeys responded serologically, indicating a poor immunogenic capacity of this vaccine compared to results obtained earlier with attenuated virus in the 80th tissue culture passage.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-02: Prevention and Treatment of Biological Warfare Casualties

Study No. -40-02: Aerogenic Immunization With Attenuated Venezuelan Equine Encephalomyelitis Virus.

Description:

To study aerogenic vaccination with attenuated Venezuelan equine encephalomyelitis virus in experimental animals.

Progress:

Aerogenic vaccination of guinea pigs and monkeys with attenuated Venezuelan equine encephalomyelitis (VEE) virus produced by National Drug Co. (ND-4) was carried out to investigate the safety and effectiveness of this product by this route. With the exception of the serologic data, the study was described in last year's Annual Report.

Dose of vaccine presented to each of 8 monkeys and hemagglutination-inhibiting (HAI) antibody responses 21 days postvaccination, 14 days post-challenge with virulent VEE virus (Colombian strain), and 14 days postre-challenge with virulent VEE virus (Trinidad strain) are displayed in Table I. (One monkey died 12 days following vaccination and the HAI titer was determined on that day).

Four monkeys demonstrated no serologic response to the vaccine. The 7 surviving monkeys were challenged with an intraperitoneal inoculation of $10^{2.75}$ $GPIPLD_{50}$ of virulent Colombian strain VEE virus 21 days after aerosol exposure to ND-4. One monkey, which had no HAI antibody response to the vaccine, died on the 2nd postchallenge day and virulent VEE virus was isolated from blood and brain mouse inoculation and identified by mouse protection test. HAI antibody response to the virulent virus was determined 14 days postchallenge in the remaining 6 animals. Two monkeys which had failed to respond serologically to the vaccine developed no HAI antibody titer. One month later, the 6 monkeys were again inoculated intraperitoneally with virulent VEE virus (10^3 $GPIPLD_{50}$ of Trinidad strain). The two animals which were previously unresponsive serologically to both the vaccine and Colombian strain developed HAI antibody.

TABLE I. HAI RESPONSE OF MONKEYS POSTVACCINATION AND POSTCHALLENGE

MONKEY NO.	PRESENTED DOSE[a] OF ATTENUATED VEE VIRUS (ND-4) $GPIPID_{50}$	RECIPROCAL HAI TITER[a] 21 days post-immunization	14 days post-challenge with virulent VEE virus (Colombian)	14 days postre-challenge with virulent VEE virus (Trinidad)
K-3-11	65,000	<10	Died day 2 post-challenge	
K-3-22	80,000	<10	<10	10,240
L-1-16	88,000	<10	320	10,240
L-1-18	48,000	<10	<10	20,480
L-1-19	68,000	320	640	1,280
L-1-27	50,000	5120	10,240	5,120
L-1-28	112,000	80 Died day 12 postvaccination		
L-2-25	64,000	640	640	5,120

a/ Preimmunization titer was <1:10 in all cases

Summary:

Aerogenic immunization of guinea pigs and monkeys was carried out with attenuated VEE virus (National Drug Co. Lot-4). Serologic responses in monkeys indicated a poor immunogenic capacity of this vaccine when compared to previous study with another strain of attenuated VEE virus, reported by Kuehne, et al (Am J Hyg 75:347, 1962)

Publications:

None

ANNUAL PROGRESS REPORT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-02:	Prevention and Treatment of Biological Warfare Casualties
Study No. -40-05:	Influence of Total Body Ionizing Irradiation on Response to Living Viral Vaccines.
Reporting Installation:	U. S. Army Medical Unit Fort Detrick, Maryland
Division:	Medical
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Author:	Nelson R. Blemly, Lt Colonel, MC
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-02:	Prevention and Treatment of Biological Warfare Casualties
Study No. 40-05:	Influence of Total Body Ionizing Irradiation on Response to Living Viral Vaccines.
Reporting Installation:	U. S. Army Medical Unit Fort Detrick, Maryland
Division:	Medical
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Author:	Nelson R. Blemly, Lt Colonel, MC
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

No work was conducted on this Study because of higher priority demands.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-02: Prevention and Treatment of Biological Warfare Casualties

Study No. -00-05: Influence of Total Body Ionizing Irradiation on Response to Living Viral Vaccines.

Description:

To assess the influence of total body x-irradiation on the immune response to an attenuated strain of Venezuelan equine encephalomyelitis virus.

Progress:

No work was conducted on this Study because of higher priority demands.

Summary:

None.

Publications:

None.

ANNUAL PROGRESS REPORT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-02:	Prevention and Treatment of Biological Warfare Casualties
Study No. -50-01:	Studies With Selected Members of Group B Arboviruses
Reporting Installation:	U. S. Army Medical Unit Fort Detrick, Maryland
Division:	Virology
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Authors:	Marie L. Miesse George A. DiGioia, PFC
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-02: Prevention and Treatment of Biological Warfare Casualties

Study No. -50-01: Studies With Selected Members of Group B Arboviruses

Reporting Installation: U. S. Army Medical Unit
Fort Detrick, Maryland

Division: Virology

Period Covered by Report: 1 July 1963 to 30 June 1964

Professional Authors: Marie L. Miesse
George A. DiGioia, PFC

Reports Control Symbol: RCS-MEDDH-288

Security Classification: UNCLASSIFIED

Langat virus, TP-21 strain has been studied in a variety of laboratory hosts and situations. Two tests to identify the virus or its antibody have been employed: the mouse neutralization and the complement-fixation tests. Only one reliable marker for presence of infectious virus by *in vivo* method is available to date, *i.e.*, death of the mouse. Monkeys, guinea pigs, embryonated eggs and tissue cultures have been asymptomatic during infection.

TP-21 multiplies well in mouse brain, moderately so in embryonated eggs and poorly in chick embryo fibroblasts and green monkey kidney cells. Serial passage in eggs and cell lines have been made in an attempt to increase viral yield or to develop other markers. To date these have not been successful. Virus has been "lost" several times in cell lines, probably because of low titers and instability on short-term storage. Experiments in other cell lines and with varying incubation temperatures are in progress.

Studies on the mechanism of cell infection are planned.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-02: Prevention and Treatment of Biological Warfare Casualties

Study No. -50-01: Studies With Selected Members of Group B Arboviruses

Description:

Investigations under this study are directed toward characterization of selected group B arboviruses. Studies are being performed to assess optimal growth conditions, stability, and immunogenic potential of the viruses, singly and in combination.

Progress:

Langat virus, TP-21 strain, is currently being characterized. The strain used in all studies was obtained from the Virology Division, Walter Reed Army Institute of Research. It was in the fifth suckling mouse brain passage. Minimal amounts of virus in the starting material necessitated several rapid brain-to-brain passages in suckling mice to obtain high-titered virus. At the 8th suckling mouse brain passage, seed material of 20% mouse brain in phosphate buffered saline with 20% rabbit serum was prepared. This material was freeze-dried on a laboratory model Aminco apparatus and stored at 4 C as standard strain reference. The 10th suckling mouse brain passage was stored at -70 C in the frozen state, as seed virus for further work. To verify the identity of the strain a neutralization test was done with the 10th passage virus using immune serum obtained from another laboratory. The serum was obtained from Dr. Winston Price, Johns Hopkins University. The serum neutralization index was in agreement with that obtained by Dr. Price's laboratory.

TP-21 in the Mouse.

When inoculated in the young adult mouse, by the intracerebral (IC) route the virus causes death starting at day 6 or 7 in the lowest dilutions and continuing through about day 12 in the higher dilutions. The same preparation causes death in the intraperitoneally (IP) inoculated mouse a day or two later. Suckling mice, inoculated by either route usually die starting day 4 or 5. The most consistent clinical manifestation of infection is a weakness or paralysis of the hind legs which may be accompanied by a "rough" coat or "bleary-eyed" appearance. Dead mice frequently are found with teeth firmly clenched to the wire

of the cage platform, suggesting a convulsive seizure just prior to death. Seizures have not been observed first-hand.

Assays of virus have been performed in 3-4 week old mice because early observations indicated no appreciable difference in final titer between assays in suckling or older animals. An experiment was performed using mice of varying ages to confirm the early observation that mouse age did not affect titers of mouse brain virus and, in addition, to determine whether mouse age affected assays of virus grown in the embryonated egg. See Table I.

TABLE I. ASSAY OF TP-21 VIRUS IN MICE OF DIFFERENT AGES

TISSUE TESTED	PASSAGE	VIRUS TITER $MICLD_{50}$ LOG_{10} Age of mice in days			
		36	29	22	2-4
Suckling Mouse Brain	10	8.0	7.8	8.63	8.75
Chick Embryo	7	5.5	5.84	5.6	5.5

Results expressed in Table I would suggest that suckling mice and 22-day old mice are slightly more sensitive in assaying TP-21 mouse brain virus than are older mice. However, assay of egg-adapted virus revealed no change in sensitivity regardless of mouse age. It was decided to continue using 3-4 week old mice for all assays.

The 50% intracerebral lethal endpoint ($MICLD_{50}$) of freshly prepared infected mouse brain suspension ranged between 8.5 and 9.0 logs/0.03 ml. Titration of these preparations by the IP route usually yielded lower values suggesting a change in virus viscerotropism as the result of continuous brain passage. Mouse median intraperitoneal infectious titers ($MIPID_{50}$) were measured by challenge of survivors of IP titrations with multiple lethal doses of virus. The route of inoculation was intracerebral.

Table II illustrates decreasing lethal titers of mice inoculated by the IP route with increasing suckling mouse brain passage. Infectious titers are higher than lethal titers by this route, suggesting a change in virulence. Lethal and infectious titers are identical when mice are inoculated IC.

Repeated assays of 10th passage standard suckling mouse brain suspension reveal that the virus has maintained essentially the same $MICLD_{50}$ titer after 6 months storage at -70 C. No auto-interference has been demonstrable in low dilution.

TABLE II. ASSAY OF TP-21 SUCKLING MOUSE BRAIN VIRUS IN 21-28 DAY OLD MICE

VIRUS PASSAGE LEVEL	ROUTE OF INOCULATION	
	Intracerebral	Intraperitoneal
	Logs virus/0.03 ml	Logs virus/0.30 ml
8	Lethal = 8.6	Lethal = 6.43
	Infectious = 8.6	Infectious = 9.0
10	Lethal = 8.6	Lethal = <4.0
	Infectious = 8.6	Infectious = 7.84

Antibody Studies.

Complement-Fixation Tests. A successful complement-fixation (CF) test was developed for the detection of TP-21 antibody. Antigens were prepared from suckling mouse brains which had about 9.0 logs infectious virus. A 20% suspension of triturated mouse brain in phosphate buffered saline was prepared. The suspension was centrifuged for 1 hr at 25,500G. The supernatant fluid was withdrawn, bottled in convenient working volumes, frozen and stored at -70 C. Usual antigen titers against 4 antibody units were 1:32 to 1:64. Four units of antigen were used in the test. Complement titers were not decreased by the presence of 4 units of antigen, and the antigen had not become anticomplementary when tested after 3 months of storage. Normal mouse brain antigens were prepared in the same manner and were included as controls in every test.

Preparation of Immune Sera. Guinea Pigs. An experiment was performed to determine conditions for development of maximal antibody against the virus and minimal antibody against the normal brain component of the infecting material. Tenth passage suckling mouse brain virus was administered and only one dose was given. Guinea pigs were inoculated by the IC route with 0.1 ml or by the IP route with 0.5 ml. Six guinea pigs were used per virus dilution and per route. None of the animals inoculated by the IP route developed fever or gross signs of infection. All of the IC guinea pigs receiving the largest dose (8.5 logs) developed fever and 2 out of 6 pigs receiving 6.5 logs developed fever. Fever was of short duration and other clinical symptoms were absent.

The animals were bled at 21 and 28 days postinoculation. CF tests were done using the mouse brain antigens previously described. None of the sera were anticomplementary. Table III shows CF antibody titers of individual guinea pigs against TP-21 antigen and normal mouse brain antigen. The sera were not adsorbed with normal mouse brain to remove the nonspecific antibody.

TABLE III. GUINEA PIG COMPLEMENT-FIXATION TITERS AGAINST TP-21 AND NORMAL BRAIN ANTIGENS

INTRACEREBRAL ROUTE						
Inoculum		Guinea Pig No.	Serum			
			21 Day		28 Day	
			Antigens			
Logs Virus	Mouse Brain		TP21	Norm	TP21	Norm
	0.1 ml					
		1	128	16	[a]	
8.5	1:10	2	64	8	64	8
		3	64	8	64	16
		4	128	32	128	16
		5	128	16	128	Neg[b]
		6	128	32		
		7	128	Neg	128	8
6.5	1:1000	8	32	Neg	32	Neg
		11	128	Neg	128	Neg
		13	8	Neg	Neg	Neg
		14	32	8	64	Neg
		15			8	Neg
5.5	1:10,000	17			Neg	Neg
		18			Neg	Neg
		19			Neg	Neg
		20			Neg	Neg
4.5	1:100,000	6/6 Negative				

a. Blank space denotes not tested.
b. Negative = <8.

It will be noted from Table III that very large doses of live virus were required to produce antibody to the virus when it was administered by the IC route. Those IC-inoculated guinea pigs which developed high level antibody also had some febrile period during the course of infection. Antibody to normal mouse brain was present in a large proportion of the animals with TP-21 antibody. Individual sera positive for TP-21 and negative for normal mouse brain were selected and pooled for use as controls. For the most part, peak titers for TP-21 occurred by the 21st day and persisted to the 28th day.

Further studies on immunization are planned to test the efficacy of TP-21 as an immunizing agent. These studies will include the use of inactivated virus grown in the embryonated egg and in tissue culture systems.

Monkeys. Two rhesus monkeys were inoculated by the IP route with TP-21 virus. One with approximately 7.0 logs mouse brain virus and the other with the same quantity of virus grown in chick embryo. Temperatures were taken twice daily for 14 days. Neither monkey had any fever during this period.

CF tests were done on pre-inoculation, 14-day and 21-day sera. Both monkeys had low level antibody (1:8) in the serum from the pre-inoculation bleeding. There was no rise in titer by the 21st day when the sera still maintained a titer of 1:8. A neutralization test was also done with these sera, with negative results. It is probable that the monkeys had been infected with a related virus such as Kyasanur Forest or yellow fever while in their native habitat. This could have afforded protection against TP-21 virus, and prevented development of antibody. In the future, monkeys will be screened for antibody to several of the group B arboviruses before being used.

Neutralization tests. A mouse IC neutralization test has been successfully employed. Pools of CF positive guinea pig sera consistently had a serum neutralization index of 4.0 or more logs. This was obtained with untreated sera or sera inactivated at 56 C for 30 minutes.

To date lack of suitable virus markers has prevented development of other neutralization techniques such as plaque inhibition or metabolic inhibition tests.

Growth of TP-21 in the Hen's Egg and Tissue Culture.

Because of the risk of causing allergic encephalitis in subjects inoculated with brain tissue, it is of importance to find a satisfactory host system other than the mouse in which to grow TP-21 virus. An ideal host system would produce virus in high yield. In addition, suitable markers such as cytopathic effect (CPE) or lethality to indicate the presence of the virus are required. To this end studies were initiated on the growth of TP-21 in the hen's egg and in tissue culture.

TP-21 in the Hen's Egg. TP-21 will replicate in the embryonated egg when inoculated by yolk sac, allantoic or chorioallantoic membrane routes. However, in all cases the virus fails to cause death of the embryo, and there are no apparent changes referable to virus infection.

Titers of approximately 6 logs have been obtained in the chick embryo. Allantoic fluid titers have been about 1 log lower. Further passages are in progress to determine whether allantoic adaptation will eventually result in higher titered virus. The stability of the virus on short-term storage as chick embryo slurry or allantoic fluid appears excellent. In contrast to some tissue culture preparations, virus stored for one month at -70 C has not dropped in titer.

TP-21 in Tissue Culture. TP-21 has been inoculated and passaged in 2 cell lines, chick embryo cells in monolayer, prepared in our laboratory and green monkey (Cercopithecus aethiops) kidney cell monolayers purchased from Microbiological Associates, Bethesda, Md. Growth rates and titers are similar if not identical for the 2 kinds of cells. No CPE has been observed. Serial passages of virus are being done to determine if viral changes due to host adaptation will result in CPE. To date plaques have not been produced in either cell line but more exhaustive studies are planned.

Table IV shows results of a typical growth experiment. Chick embryo cell monolayers were inoculated with varying dilutions of virus and incubated at 35 C. On days 3, 4, 5, and 6 the supernatant fluids were removed and replaced with fresh media. The supernates were titrated in mice.

TABLE IV. ASSAY[a] OF TP-21 VIRUS GROWN IN CHICK EMBRYO CELLS

INOCULUM TP-21, CF1[b] PASSAGE DILUTION	VIRUS TITER MICLD$_{50}$ BY DAY OF HARVEST			
	3	4	5	6
10^{-1}	3.10	2.83	N.D.[c]	N.D.
10^{-2}	2.60	3.63	3.5	2.35
10^{-3}	1.87	4.0	3.75	3.63
10^{-4} to 10^{-6}	Neg	Neg	Neg	Neg

a. Assayed for lethality in intracerebrally inoculated mice and expressed in logs.
b. First passage in chick embryo cells.
c. ND = Not done.

Virus yields were very low, and the cells continued to produce low levels of infectious virus until about the 6th day when both infected and control cells began to die.

There has been considerable difficulty in maintaining the virus in serial passage in monkey kidney cells. On several occasions it has been necessary to return to early passage material after mouse titrations revealed that the virus had been "lost". These unsuccessful passages were associated with storage of the virus over short periods when the commercial supplier had failed to deliver the usual monkey kidney cell supply. An experiment was performed to determine if short-term storage and absence of protective protein in the stored material might cause rapid inactivation of the virus. Five bottles of monkey kidney cells were inoculated with virus. After a short period for viral adsorption the bottles were overlaid with [illegible]. Two bottles received Mixture 199 with no serum. The remaining 3 bottles received Mixture 199 with 1%, 2% and 3% volumes of whole calf serum respectively. The media were not changed during the course of the experiment. On days 4 and 5 samples were taken from each bottle and titrated in mice. The samples were frozen and stored for 11 days at -70 C. Samples from one of the bottles which received 199 only as media, were treated with calf serum to give a final 2% concentration just prior to storage. Results of this experiment are seen in Table V. From this chart it can be seen that the most stable virus was from the culture where only media 199 was used during growth and serum was added just prior to storage. When no serum was used during growth or storage, or when serum was in the media during virus replication there was a decrease of titer. It would appear from this experiment that fresh, undegraded serum protein serves well to stabilize TP-21 virus on short-term storage.

TABLE V. EFFECT OF SHORT-TERM STORAGE AND SERUM ON TP-21 VIRUS

	IMMEDIATE TITRATION		11-DAY STORAGE	
	Day of Sample		Day of Sample	
MEDIA	4	5	4	5
199	3.5[a]	3.5	2.3	<2.0
199[b]	3.25	3.5	3.3	3.4
199_{99} $Calf_1$	2.64	3.25	2.25	X[c]
199_{98} $Calf_2$	3.42	3.5	2.75	X
199_{97} $Calf_3$	3.25	4.62	2.6	X

a. Mouse intracerebral median lethal dose expressed in logs.
b. 2% Calf serum added before storage.
c. Not calculated. No deaths at 10^{-2} but deaths in weaker dilutions.

After storage, virus grown for 5 days in cells overlaid with serum-containing media did not produce the expected death pattern in mice when inoculated in serial log dilutions. Mice inoculated with the lowest dilution of 10^{-2} survived, whereas those in subsequent dilutions through 10^{-4} died within the period of time previously established for TP-21 virus. This may be the result of interference between high levels of nonviable virus accrued during incubation and storage, and surviving live virus. These results are entirely consistent with those found in classic studies of auto-interference first described with influenza virus. Further definitive studies would be required to establish the exact cause of the apparent interference.

Other studies of TP-21 in cell systems are in progress. Hamster kidney cells are in use. Experiments are being done to determine optimal growth temperatures. Future experiments are planned to study the mechanisms of virus infection of the cell.

Summary and Conclusions:

Langat virus, TP-21 strain, will grow well in the mouse brain but poorly in other laboratory hosts with the methods employed. Tissue culture virus is unstable on short-term storage in the frozen state, without addition of whole serum before freezing. High doses of live virus are necessary to produce complement-fixation or neutralizing antibody. Experiments with inactivated virus have not been performed.

Studies are in progress to increase viral yields and to uncover additional markers of viral activity.

Publications:

None.

ANNUAL PROGRESS REPORT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-03:	Laboratory Identification of Biological Warfare Agents
Study No. -00-01:	Identification of Airborne Particulates by Fluorescent Antibody Techniques
Reporting Installation:	U. S. Army Medical Unit Fort Detrick, Maryland
Division:	Pathology
Period Covered by Report:	1 July 1963 through 30 June 1964
Professional Author:	Robert F. Jaeger
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-03:	Laboratory Identification of Biological Warfare Agents
Study No. -00-01:	Identification of Airborne Particulates by Fluorescent Antibody Techniques
Reporting Installation:	U. S. Army Medical Unit Fort Detrick, Maryland
Division:	Pathology
Period Covered by Report:	1 July 1963 through 30 June 1964
Professional Author:	Robert F. Jaeger
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

No investigations were conducted during the reporting period.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C522401A096-03: Laboratory Identification of Biological Warfare Agents

Study No. -00-01: Identification of Airborne Particulates by Fluorescent Antibody Techniques

Description:

To determine whether impactors and other samplers combined with fluorescent antibody procedures will provide for detection of airborne infective agents.

Progress:

No investigations were conducted during the reporting period.

Summary and Conclusions:

None.

Publications:

None.

ANNUAL PROGRESS REPORT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-03:	Laboratory Identification of Biological Warfare Agents
Study No. -00-02:	Identification of Microbial Pathogens by Fluorescent Antibody Techniques
Reporting Installation:	U. S. Army Medical Unit Fort Detrick, Maryland
Division:	Pathology
Period Covered by Report:	1 July 1963 through 30 June 1964
Professional Authors:	Sections I and II Robert F. Jaeger Warren Sanborn, Lieutenant, USN Robert F. Robertson, HMC, USN
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-03:	Laboratory Identification of Biological Warfare Agents
Study No. -00-02:	Identification of Microbial Pathogens by Fluorescent Antibody Techniques
	Section I. Optimum pH Values for Methanol Precipitation of Globulin Fractions from Sera of Several Animal Species
Reporting Installation:	U. S. Army Medical Unit Fort Detrick, Maryland
Division:	Pathology
Period Covered by Report:	1 July 1963 through 30 June 1964
Professional Authors:	Section I Robert F. Jaeger Warren Sanborn, Lieutenant, USN Robert F. Robertson, HMC, USN
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

Sera from burros, chickens, rabbits and swine were fractionated by the Dubert methanol method, using a single precipitation at -10 C. Total proteins were calculated by the Biuret method; paper electrophoresis was employed to evaluate the globulin fractions. No significant variations in total proteins were observed in any single serum sample over a pH range of 4.9-7.6. The optimal pH for maximum yield of globulins was 7.0, with only minor variations in the values obtained between 7.0 and 7.3.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-03: Laboratory Identification of Biological Warfare Agents

Study No. -00-02: Identification of Microbial Pathogens by Fluorescent Antibody Techniques

Section I. Optimum pH Values for Methanol Precipitation of Globulin Fractions from Sera of Several Animal Species

Description:

To study the adaptation of fluorescent antibody techniques, found to be useful in identifying such agents as Rift Valley fever virus in tissues to the demonstration of other microbial pathogens in animal tissues.

Progress:

Samples of normal burro, chicken, rabbit and swine sera were obtained from clinically normal animals. Portions of these sera were separately fractionated by the Dubert methanol method (one precipitation at -10 C) at pH values ranging from 4.9-7.6. Following fractionation, the total protein of each test sample was calculated by the Biuret method. Globulin fractions were determined by paper electrophoresis.

The total protein determinations were remarkably consistent, no matter which pH was used during fractionation. The electrophoretic patterns indicated the optimum pH for maximum globulin return from the Dubert methanol fractionation method was 7.0, and that, in the range 7.0-7.3, little variation was observed in results.

Summary and Conclusions:

During methanol fractionation of sera by the method of Dubert, the greatest globulin return from the procedure was obtained at pH 7.0.

Publications:

None.

ABSTRACT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-03:	Laboratory Identification of Biological Warfare Agents
Study No. -00-02:	Identification of Microbial Pathogens by Fluorescent Antibody Techniques
	Section II. Physiological Conditioning of Roosters to Produce Higher Titer Antisera
Reporting Installation:	U. S. Army Medical Unit Fort Detrick, Maryland
Division:	Pathology
Period Covered by Report:	1 July 1963 through 30 June 1964
Professional Authors:	Section II Robert F. Jaeger Warren Sanborn, Lieutenant, USN Robert F. Robertson, HMC, USN
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

Pure silica powder, 5μ particle size, was injected into roosters via the intravenous route. Thirty days after injection of the silica powder, separate groups of these treated animals were challenged with the SCHU-S4 strain of Pasteurella tularensis and Venezuelan equine encephalomyelitis virus, Trinidad strain. Untreated control roosters were also challenged at the same time. After appropriate intervals, both treated and untreated animals were bled out, the sera titrated for antibody levels, and portions conjugated with fluorescein isothiocyanate. No significant differences were observed between test groups in production of antibody. Further, the sera from silica-treated roosters was universally cloudy and generally unsatisfactory for research use when conjugated with fluorescence.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-03: Laboratory Identification of Biological Warfare Agents

Study No. -00-02: Identification of Microbial Pathogens by Fluorescent Antibody Techniques

Section II. Physiological Conditioning of Roosters to Produce Higher Titer Antisera

Description:

To study the adaptation of fluorescent antibody techniques, found to be useful in identifying such agents as Rift Valley fever virus in tissues to the demonstration of other microbial pathogens in animal tissues.

Progress:

Pernis and Paronetto (Proc Soc Exp Biol Med 110:390, 1962) have indicated that injections of pure silica powder may effectively increase production of antibody in certain species of animals. In order to determine whether this method is effective in increasing the antibody titer in rooster sera against Pasteurella tularensis and Venezuelan equine encephalomyelitis (VEE), roosters were given 70 µg/kg of pure silica powder, 5µ particle size, via the intravenous route. Thirty days following the completion of the silica injection regimen, separate groups of the fowl were challenged with the SCHU-S4 strain of P. tularensis and VEE virus, Trinidad strain. Nontreated controls were also challenged at the same time.

After appropriate intervals, the roosters were bled out, the sera were fractionated and portions of the final product conjugated with fluorescein isothiocyanate. Total proteins were measured and the globulin fractions calculated by proper electrophoresis.

The average antibody titers produced in the silica-treated roosters were no higher than those in nontreated control animals. Total proteins were similar and the percentage values of the globulin fractions were not altered by the prechallenge treatment. Further, it was extremely difficult to clarify the conjugated portions from the treated animals and in general, these tagged antisera gave poor results under experimental circumstances.

Summary and Conclusions:

Prechallenge treatment of roosters with silica powder failed to stimulate an increase in antibody titer, when these animals were later challenged with P. tularensis and Trinidad strain VEE virus. The silica also interfered with the conjugation procedures and an unsatisfactory tagged antiserum resulted.

Publications:

None.

ANNUAL PROGRESS REPORT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-03:	Laboratory Identification of Biological Warfare Agents
Study No. -00-04:	Detection of Circulating Antigens by Chromatographic Methods
Reporting Installation:	U.S. Army Medical Unit Fort Detrick, Maryland
Division:	Physical Sciences Division
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Author:	Irving Gray, Colonel, MSC
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-03:	Laboratory Identification of Biological Warfare Agents
Study No. -00-04:	Detection of Circulating Antigens by Chromatographic Methods
Reporting Installation:	U.S. Army Medical Unit Fort Detrick, Maryland
Division:	Physical Sciences Division
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Author:	Irving Gray, Colonel, MSC
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

Because of higher priority work in the division, this study has been terminated. It will be carried out as part of a research contract with Georgetown University, Washington, D.C.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biolcgical Warfare (U)

Task No. 1C622401A096-03: Laboratory Identification of Biological Warfare Agents

Study No. -00-04: Detection of Circulating Antigens by Chromatographic Methods

Description:

To develop chromatographic methods for the identification of circulating antigens.

Progress:

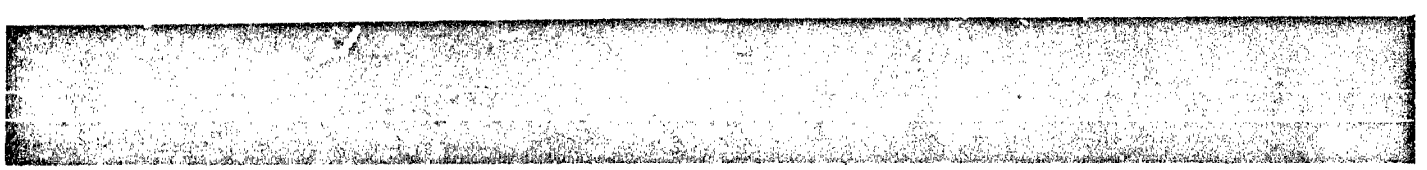
Because of higher priority work in the division, this study has been terminated. It will be carried out as part of a research contract with Georgetown University, Washington, D.C.

Publications:

None.

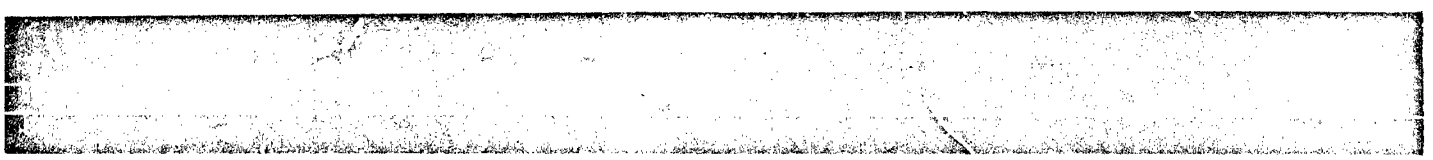
Presentations:

None.



ANNUAL PROGRESS REPORT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-03:	Laboratory Identification of Biological Warfare Agents
Study No. -00-05:	Diagnosis of Viral Infections with Homologous Bone Marrow Cultures
Reporting Installation:	U.S. Army Medical Unit Fort Detrick, Maryland
Division:	Medical
Professional Authors:	Thomas J. Smith, Major, MC Robert W. McKinney, Major, MSC William D. Sawyer, Major, MC
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED



ABSTRACT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-03: Laboratory Identification of Biological Warfare Agents

Study No. -00-05: Diagnosis of Viral Infections with Homologous Bone Marrow Cultures

Reporting Installation: U.S. Army Medical Unit
Fort Detrick, Maryland

Division: Medical

Period Covered by Report: 1 July 1963 to 30 June 1964

Professional Authors: Thomas J. Smith, Major, MC
Robert W. McKinney, Major, MSC
William D. Sawyer, Major, MC

Reports Control Symbol: RCS-MEDDH-288

Security Classification: UNCLASSIFIED

In vitro culture of bone marrow obtained relatively late in the course of disease in both man and monkeys has resulted in isolation of VEE virus not detected by conventional procedures. Viable marrow cells served as both the source of virus and as susceptible tissue supporting virus multiplication. It is suggested that this method may have application in the diagnosis of other viral diseases.

A manuscript has been written which will be submitted for publication.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-03: Laboratory Identification of Biological Warfare Agents

Study No. -00-05: Diagnosis of Viral Infections with Homologous Bone Marrow Cultures

Description:

To develop a diagnostic system in which bone marrow serves as both a clinical specimen and a host system for in vitro virus propagation.

Progress:

A system of in vitro cultivation of bone marrow, employing monolayer cultures of marrow cells alone and marrow cells in combination with L cells, was described in the FY 1963 Annual Report. These studies have been extended employing the same techniques.

Venezuelan equine encephalomyelitis (VEE) infection in monkeys following subcutaneous inoculation of 1000 $MIPLD_{50}$ of the virulent virus (Trinidad strain) was accompanied regularly by viremia (Table I). By testing in mice, virus was demonstrated in all blood and bone marrow samples obtained during the first 4 days of infection, but none after day 5.

TABLE I. PRESENCE OF VEE VIRUS IN MONKEY BLOOD AND BONE MARROW AS DETERMINED BY MOUSE INOCULATION

	NO. POSITIVE/NO. SPECIMENS TESTED Day of Infection						
Specimen	1	2	3	4	5	6	7
Blood	2/2	2/2	6/6	6/6	2/8	0/7	0/8
Bone marrow	2/2	2/2	4/4	1/1	1/5	0/5	0/8

In 5 of 12 monkeys, however, bone marrow culture resulted in isolation of virus in one or both cultural systems relatively late in the course of infection, after viremia had subsided (Table II). With the exception of the day 5 marr[illegible] from monkey No. 10A-9, direct mouse inoculation of the marrow samples failed to demonstrate virus and all corresponding peripheral blood samples were negative when tested in mice and L cell cultures.

TABLE II. VEE VIRUS CONTENT OF TISSUE CULTURE FLUID AS DETERMINED BY MOUSE INOCULATION

MONKEY NO.	DAY OF INFECTION	HEMAGGLUTINATION-INHIBITION ANTI-BODY TITER	TISSUE CULTURE FLUID		
			Day of incubation	Marrow culture	Marrow - L cell culture
C-20	7	1:80	1	-	+
			2	-	+
			3	-	+
			4	+	+
			7	+	+
B-336	6	Not done	1	-	-
			4	-	+
	7	1:80	3	+	-
			5	+	-
N-11	6	Not done	1	+	+
			5	+	+
			7	+	+
7A-11	6	1:80	1	+	+
			2	-	+
			4	-	+
			5	-	+
10A-9	5	1:10	1	+	Not done
			2	+	
			3	+	
			4	+	

In the cultural system employing marrow cells alone, virus not only persisted but multiplied to relatively high titer. In general, marrow cells obtained from monkeys early in the infection survived *in vitro* for only several days, and inoculation of these early specimens containing virus in high titer into L cell cultures resulted in gross cytopathic effect (CPE) in 48-72 hours. Marrow cells obtained after day 4 of infection could, with few exceptions, be maintained *in vitro* for at least 1 week. In some instances cultures of marrow cells alone or in combination with L cells remained viable for almost a month; virus was produced continuously during this period. Such chronically infected cultures were noteworthy in that VEE virus usually produces marked CPE and death of L cells in 48-72 hours. Mouse inoculation of virus harvested from these long term cultures demonstrated no viral attenuation.

A portion of each of the marrow samples obtained after day 5 of infection was subjected to 1 or 2 freeze-thaw cycles to disrupt cells prior to inoculation into L cell culture. Experience with the virus in this laboratory has shown it to be stable upon such treatment. Supernatant fluids of cultures inoculated with these materials contained no virus. Thus it would appear that viable marrow cells were required for virus multiplication to occur, in spite of the fact that L cells are known to be highly susceptible to infection with VEE virus.

The investigative use of VEE virus as an antineoplastic agent in man, reported by Tigertt and co-workers (Cancer 15:628, 1962) afforded the opportunity to extend these studies to a small series of patients. A single bone marrow specimen was obtained from each of 6 patients with lymphomatous disease 8-13 days after subcutaneous inoculation of the attenuated strain. Direct mouse inoculation failed to demonstrate virus in any of these specimens. In one instance, however, *in vitro* incubation of a marrow sample obtained on postinoculation day 8 resulted in multiplication and demonstration of the virus. Supernatant fluids removed from the marrow culture after 24 and 48 hours of *in vitro* incubation protected mice against challenge with virulent virus. The fluids were of low virus titer, containing slightly greater than 100 $MIPID_{50}$/ml at 48 hr. The cultures consisted of marrow cells only; the marrow-L cell monolayer was not employed. At a later date the same marrow sample was inoculated into an L cell culture after prior freeze-thaw cycles; no virus was recovered.

As objective evidence of tumor regression was noted in some patients infected with the attenuated strain of virus and in the hope that the Trinidad VEE strain would exert a more profound antitumor effect, 2 patients received this virus. Both had far-advanced Hodgkin's disease which was progressive despite all other forms of therapy. Both tolerated the infection well and neither exhibited evidence of encephalitis. In one, a dramatic, though short-lived, remission of the Hodgkin's disease occurred. Bone marrow aspirates and samples of peripheral blood were obtained from this patient on postinoculation days 2, 6, 10 and 18 and tested for virus content (Table III). Day 2 and day 6 marrow contained virus in lower titer than the corresponding peripheral blood. On days 10 and 18 both blood and bone marrow were negative upon direct mouse inoculation.

TABLE III. VEE VIRUS CONTENT OF BLOOD, BONE MARROW AND BONE MARROW CULTURE FLUIDS - PATIENT CD

POST-INOCULATION DAY	TITER OF BLOOD[a]	TITE. OF MARROW[a] PRIOR TO IN VITRO CULTIVATION	DAYS OF IN VITRO CULTIVATION	VIRUS TITER[a] OF SUPRENATANT FLUID Marrow cells alone	Marrow + L cells
2	8.5	5.1	1	3.5	4.9
			2	2.7	5.9
			3	1.8	5.0
			4	2.5	5.8
6	4.5	2.9	1	1.0	3.5
			2	1.0	6.9
			3	3.1	9.5
			4	4.1	8.7
10	Negative	Negative	1	0.8	0.8
			2	1.8	Negative
			3	4.1	Negative
			4	5.9	1.0
18	Negative	Negative	1	Negative	Negative
			2	"	"
			3	"	"
			4	"	"
			5	"	4.7
			6	"	7.5

a/ Log_{10} - $MIPLD_{50}$/ml

No virus was recovered from L cell cultures inoculated with peripheral blood obtained on postinoculation days 10 and 18. Hemagglutination-inhibiting antibody titers of day 10 and day 18 sera were respectively 1:40 and 1:10,240. The latter serum neutralized greater than 10^6 $MIPLD_{50}$ of VEE virus. Virus was recovered from both day 10 and day 18 marrow by marrow culture. Five days of in vitro incubation of the day 18 marrow were required before virus was demonstrated. No CPE occurred in this culture, and the infected cells were maintained for more than 1 month. After repeated freeze-thaw cycles portions of these marrows (days 10 and 18) were incubated with L cells and, as was the case with the monkey marrows, no virus was recovered. This again indicates that marrow cells must remain viable in vitro to allow for virus multiplication.

Summary and Conclusions:

In vitro culture of bone marrow obtained relatively late in the course of disease in both man and monkeys has resulted in isolation of VEE virus not detected by conventional procedures. Viable marrow cells served as both the source of virus and as susceptible tissue supporting virus multiplications. It is suggested that this method may have application in the diagnosis of other viral diagnosis.

Publications:

None.

ANNUAL PROGRESS REPORT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-03:	Laboratory Identification of Biological Warfare Agents
Study No. -00-06:	Study of Selected Arthropod Vector-Host Relationships by Immunologic Methods
Reporting Installation:	U. S. Army Medical Unit Fort Detrick, Maryland
Division:	Virology
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Author:	Warren R. Brunton, Captain, VC
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-03: Laboratory Identification of Biological Warfare Agents

Study No. -00-06: Study of Selected Arthropod Vector-Host Relationships by Immunologic Methods

Reporting Installation: U. S. Army Medical Unit
Fort Detrick, Maryland

Division: Virology

Period Covered by Report: 1 July 1963 to 30 June 1964

Professional Author: Warren R. Brunton, Captain, VC

Reports Control Symbol: RCS-MEDDH-288

Security Classification: UNCLASSIFIED

The use of guinea pigs sensitized to heterologous animal protein for the purpose of identifying the source of arthropod blood meals does not appear feasible.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-03: Laboratory Identification of Biological Warfare Agents

Study No. -00-06: Study of Selected Arthropod Vector-Host Relationships by Immunologic Methods

Description:

To investigate the possible value of using guinea pigs sensitized to heterologous animal protein for the dual purpose of identifying the source of arthropod blood meals and for isolation of agents transmitted by these arthropods.

Progress:

In preliminary experiments guinea pigs rendered hypersensitive to horse serum were employed. Sensitization was accomplished by the inoculation of horse serum incorporated in Freund's complete adjuvant.

Initially Aedes triseriatus mosquitoes were fed horse serum by the hanging drop technique in order to determine whether serum proteins could be detected following ingestion.

On days 0, 2, 4, 8, and 16 after feeding, 10 mosquitoes were triturated, and the supernatant fluid was inoculated intradermally into the previously sensitized guinea pigs. No visible skin reactions occurred. It appears that failure to obtain reactions was related to the degree of sensitization. This conclusion is supported by the fact that inoculation of relatively large amounts of horse serum elicited skin responses in the same animals.

Repeated attempts to increase the sensitivity of the system were unsuccessful. No additional experiments are planned.

Summary and Conclusions:

The use of guinea pigs sensitized to heterologous animal protein for the purpose of identifying the source of arthropod blood meals does not appear feasible.

Publications:

None.

ANNUAL PROGRESS REPORT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-03:	Laboratory Identification of Biological Warfare Agents
Study No. -00-07:	Preparation and Use of Specific Fluorescent Antibodies
Reporting Installation:	U. S. Army Medical Unit Fort Detrick, Maryland
Division:	Bacteriology and Immunology Branch Armed Forces Institute of Pathology Walter Reed Army Medical Center Washington, D. C.
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Author:	Elmer F. Chaffee, Colonel, MSC
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-03: Laboratory Identification of Biological Warfare Agents

Study No. -00-07: Preparation and Use of Specific Fluorescent Antibodies

Reporting Installation: U. S. Army Medical Unit
Fort Detrick, Maryland

Division: Bacteriology and Immunology Branch
Armed Forces Institute of Pathology
Walter Reed Army Medical Center
Washington, D. C.

Period Covered by Report: 1 July 1963 to 30 June 1964

Professional Author: Elmer F. Chaffee, Colonel, MSC

Reports Control Symbol: RCS-MEDDH-288

Security Classification: UNCLASSIFIED

Work carried out under this study is reported in the FY 1964 Annual Progress Report of the Armed Forces Institute of Pathology (RCS-MEDDH-288).

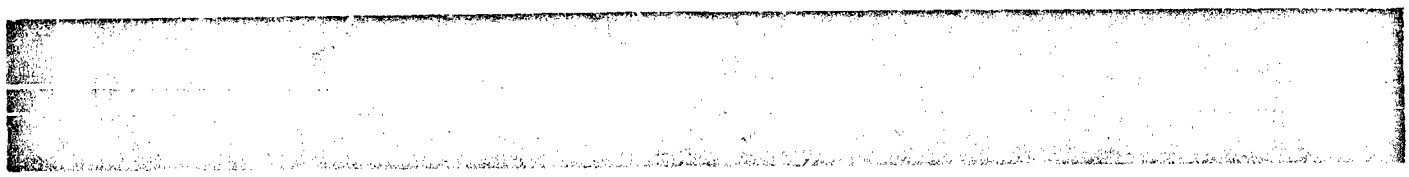
BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-03: Laboratory Identification of Biological Warfare Agents

Study No. -00-07: Preparation and Use of Specific Fluorescent Antibodies

Work carried out under this study is reported in the FY 1964 Annual Progress Report of the Armed Forces Institute of Pathology (RCS-MEDDH-288).



ANNUAL PROGRESS REPORT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-03:	Laboratory Identification of Biological Warfare Agents
Study No. -10-03:	Definition of Nutritional Requirements, Specific Metabolic Activities and Determinants of Virulence of Pasteurella tularensis
Reporting Installation:	U. S. Army Medical Unit Fort Detrick, Maryland
Division:	Bacteriology
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Authors:	Martha K. Ward, Captain, USPHS Hugh B. Tresselt, Ph.D.
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-03: Laboratory Identification of Biological Warfare Agents

Study No. -10-03: Definition of Nutritional Requirements, Specific Metabolic Activities and Determinants of Virulence of Pasteurella tularensis

Reporting Installation: U. S. Army Medical Unit
Fort Detrick, Maryland

Division: Bacteriology

Period Covered by Report: 1 July 1963 to 30 June 1964

Professional Authors: Martha K. Ward, Captain, USPHS
Hugh B. Tresselt, Ph.D.

Reports Control Symbol: RCS-MEDDH-288

Security Classification: UNCLASSIFIED

No progress to report at this time.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-03: Laboratory Identification of Biological Warfare Agents

Study No. -10-03: Definition of Nutritional Requirements, Specific Metabolic Activities and Determinants of Virulence of Pasteurella tularensis

Description:

1. To define the specific nutritional requirements for optimal growth of virulent and avirulent strains of Pasteurella tularensis.

2. To identify basic differences and mechanisms involved in the metabolic activities of fully virulent and avirulent strains.

3. To define, in so far as possible, the determinants of virulence in this species.

Progress:

No progress to report at this time.

Publications and/or Presentations:

None.

ANNUAL PROGRESS REPORT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-03:	Laboratory Identification of Biological Warfare Agents
Study No. -10-05:	Serologically Demonstrable Response to Antigens of Pasteurella tularensis
Reporting Installation:	U. S. Army Medical Unit Fort Detrick, Maryland
Division:	Bacteriology
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Authors:	Martha K. Ward, Captain, USPHS Margaret L. Huff
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-03: Laboratory Identification of Biological Warfare Agents

Study No. -10-05: Serologically Demonstrable Response to Antigens of Pasteurella tularensis

Reporting Installation: U. S. Army Medical Unit
Fort Detrick, Maryland

Division: Bacteriology

Period Covered by Report: 1 July 1963 to 30 June 1964

Professional Authors: Martha K. Ward, Captain, USPHS
Margaret L. Huff

Reports Control Symbol: RCS-MEDDH-288

Security Classification: UNCLASSIFIED

No progress to report at this time.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-03: Laboratory Identification of Biological Warfare Agents

Study No. -10-05: Serologically Demonstrable Response to Antigens Of *Pasteurella tularensis*

Description:

To develop *in vitro* procedures which may be used to determine the degree of protection against infection afforded by vaccination against tularemia or prior infection.

Progress:

No progress to report at this time.

Publications and/or Presentations:

None.

ANNUAL PROGRESS REPORT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-03:	Laboratory Identification of Biological Warfare Agents
Study No. -20-01:	Characterization of Strains of Bacillus anthracis that Differ in Biologic Activity
Reporting Installation:	U. S. Army Medical Unit Fort Detrick, Maryland
Division:	Bacteriology
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Authors:	Virginia G. McGann, Ph.D. Elizabeth O. Roberts, Ph.D.
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-03: Laboratory Identification of Biological Warfare Agents

Study No. -20-01: Characterization of Strains of Bacillus anthracis that Differ in Biologic Activity

Reporting Installation: U. S. Army Medical Unit
Fort Detrick, Maryland

Division: Bacteriology

Period Covered by Report: 1 July 1963 to 30 June 1964

Professional Authors: Virginia G. McGann, Ph.D.
Elizabeth O. Roberts, Ph.D.

Reports Control Symbol: RCS-MEDDH-288

Security Classification: UNCLASSIFIED

Several areas were explored in the present studies on development of criteria for in vitro identification of strains of Bacillus anthracis. Cultural reactions and virulence of mutants isolated after challenge of treated animals were investigated. Methods for in vitro detection of sensitivity to penicillin were compared and an attempt was made to distinguish between strains by the effect of antiserum on growth. A considerable effort was made to determine the germination, growth and sporulation requirements of several strains in a minimal, chemically-defined medium. Results of these experiments will be reevaluated because recent work indicates the presence of impurities in a component of the medium.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-03: Laboratory Identification of Biological Warfare Agents

Study No. -20-01: Characterization of Strains of Bacillus anthracis that Differ in Biologic Activity

Description:

To develop criteria for in vitro identification of strains or mutants of Bacillus anthracis that differ in biologic activity.

To determine mechanisms of in vivo production of mutants.

Progress:

Investigations were continued with a number of wild strains of Bacillus anthracis and with mutants isolated at autopsy from immunized guinea pigs and from a monkey inadequately treated with penicillin. Virulence, penicillin sensitivity and growth in complex and in chemically-defined media were studied. The following strains and mutants were used: (1) wild strains V1b-189, NH-6, 95, 107, 108, 116, 992, 979, 1133 and 7287; (2) mutants of V1b-189 from the penicillin-treated monkey: 4-8 and 10-24; (3) mutants of V1b-189 from immunized guinea pigs:5C, 201A, 202B, 203C and 205A; and (4) mutants of NH-6 from immunized guinea pigs: 101A, 101C, 105C, 114C and 308C.

Virulence Studies with Mutants. Homogeneous spore suspensions were prepared from representative mutant strains. All strains produced capsules when grown on bicarbonate agar in an atmosphere of CO_2, but in many instances colonial appearance was markedly different from that of the parent strains. In general, all mutants grew more slowly and formed more opaque colonies; some mutants produced colonies with a creamy consistency and some, colonies with a faint yellowish color. The unusual colonial appearance of these strains suggested that the mutants might be less virulent than the parent strains. Consequently, representative strains were selected for virulence titrations in guinea pigs.

Virulence and some cultural characteristics of parent and mutant strains are summarized in Table I. Challenge suspensions were prepared at 3 dose levels for each strain: 10, 50 and 250 spores/dose. Groups of 5 guinea pigs of the Hartley strain (450 ± 50 gm), identified by tatoo, were injected subcutaneously with 0.5 ml amounts of each suspension. Animals were observed twice daily for 21 days, but all deaths occurred within 3 to 7 days. Spleens were removed at autopsy from most of the animals that died and were used for

TABLE I. VIRULENCE AND CULTURAL CHARACTERISTICS OF PARENT AND MUTANT STRAINS OF B. ANTHRACIS AT TIME OF ISOLATION

PARENT STRAIN	MUTANT STRAIN[a]	GUINEA PIG LD_{50} (Spores)	COLONY TYPE	PENICILLIN RESISTANCE	CLONE LYSIS (Minimal Medium)	DELAYED HEMOLYSIS ON SHEEP BLOOD AGAR PLATES
V1b-189	-	4	Somewhat opaque	-	-	Weak
	PENICILLIN					
	10-24	20	Opaque	+	-	Weak
	4-8	240	Opaque, yellow	+	-	-
	IMMUNE					
	201A	10	Opaque	-	-	Weak
	203C	15	Opaque, rimmed	+	+	Strong
	5C	20[b]	Pinpoint	-	-	-
	205A	35	Opaque, creamy	+	-	-
	202B	50[b]	Pinpoint	-	-	Weak
NH-6	-	35	Translucent	-	+	-
	IMMUNE					
	114C	9	Opaque	+	+	-
	101C	11	Translucent	+	+	-
	105C	16	Opaque	+	+	Weak
	308C	15	Opaque, small	+	+	Variable
	101A	50[b]	Opaque, creamy, yellow	-	-	Variable

a. Penicillin: strains isolated from a monkey inadequately treated with penicillin.
Immune: strains isolated from guinea pigs immunized with protective antigen.

b. Long chains of organisms in spleen impressions.

preparation of impression smears and for culture on nutrient agar and bicarbonate agar. In every instance, characteristics of all organisms recovered after death of the animal were the same as those of the corresponding challenge suspensions. Extremely long chains of organisms were observed in spleen impressions from animals challenged with 2 mutants (5C and 202B) of strain V1b-189 and 1 mutant (101A) of strain NH-6; the paucity of bacilli in these smears suggested that the concentrations of organisms at time of death was relatively low. High concentrations of the usual short

chains of bacilli were found in impression smears from animals challenged with all other mutants; in many instances, however, the bacilli were more variable in size or staining reaction than those of the parent strains. Challenge of all survivors at 21 days with 1,000 spores of strain V1b-189 resulted in death within 3 to 5 days, indicating the absence of an immune response in animals that survived a dose of 10-250 spores of the mutant strains.

There was some evidence that relative virulence of strains for mice might differ from virulence for guinea pigs. In studies designed for another purpose, groups of mice were challenged subcutaneously with 100 spores of each of 4 strains. Mortality for mice did not correspond to expected mortalities, as predicted from dose in terms of guinea pig LD_{50} (Table II). Virulence titrations will be done in mice with a number of strains to investigate the possibility of using host response as a means of distinguishing strain differences.

TABLE II. MOUSE SURVIVAL RATIO AND MEAN TIME TO DEATH AFTER SUBCUTANEOUS CHALLENGE WITH STRAINS OF B. ANTHRACIS (Challenge dose, 100 spores)

STRAIN	DOSE IN TERMS OF GPLD$_{50}$	MORTALITY RATIO No. Dead/No. Challenged	MEAN TIME TO DEATH (Days)
V1b-189	25.0	18/20	2.6
NH-6	3.0	20/20	2.3
10-24	5.0	13/20	3.5
4-8	0.4	18/20	4.1

Cultural reactions gave little indication of relative virulence. Animals challenged with strains that grew slowly in vitro (10-24, 5C and 202B) survived somewhat longer than those challenged with more rapidly growing strains of the same or lower virulence. Cultures with a creamy consistency of growth or a slight yellowish color (4-8, 205A and 101A) were somewhat reduced in virulence; but 7 of the 12 strains appeared to be as virulent for guinea pigs as the parent strains. All cultures were encapsulated when grown on bicarbonate agar in an atmosphere of CO_2 but one mutant (202B) was unable to grow under these conditions unless vegetative cells or high concentrations of spores were used as an inoculum. On minimal, defined medium colonies of several mutants, especially those of NH-6 origin, appeared lysed as if by bacteriophage.

At the time the virulence titrations were done, 2 mutants of strain V1b-189 from immunized guinea pigs (205A, 203C) and 4 mutants of strain NH-6 (101C, 105C, 114C, 308C) were resistant to penicillin. If 14-hr growth on bicarbonate agar was overlaid with agar containing sufficient penicillin to give 100 units/ml of medium, 10-30% of the colonies of these resistant strains (original inoculum, 100 spores) continued to grow whereas colonies of the sensitive parent strains were lysed by 1 unit penicillin/ml. In contrast to previous work with strains 4-8 and 10-24, no attempt was made at this time to establish populations of uniformly high resistance. Approximately a year later additional studies, described below, were initiated to investigate outgrowth of spore inocula in the presence of penicillin. When spore suspensions of the guinea pig mutant strains were re-examined, no resistance to penicillin could be demonstrated. This loss of resistance may be associated with a decrease in spore count that also occurred during storage. Experiments with fresh isolates obtained after challenge of another group of inadequately immunized guinea pigs may provide an explanation for the altered activity of the present suspensions. A portion of this material has been included in a report submitted for publication under Study No. 02-20-01.

Studies with Penicillin. Previous investigations on the effect of penicillin were confined to studies with encapsulated vegetative cells, in the belief that such cells more closely approximated the in vivo state of the organism. By overlay techniques it was possible to select encapsulated strains with uniformly high resistance to penicillin. Strains 4-8 and 10-24 were selected by this method and have remained stable for several years. Additional studies were undertaken to compare other aspects of the growth response of such resistant strains with those of typical sensitive strains in the presence of penicillin.

In the standard overlay test, bicarbonate agar plates were inoculated with approximately 100 spores, incubated for 14 hr in an atmosphere of CO_2, and overlaid with 2% Bacto agar containing sufficient penicillin to give 100 units/ml medium. Under these conditions colonies of sensitive strains were lysed whereas colonies of resistant strains continued to grow. If nutrient agar was substituted for bicarbonate agar and the plates incubated in air for 14 hr before overlay, colonies of sensitive strains as well as those of resistant strains, were able to continue growth in the presence of penicillin. By decreasing the time between inoculation and overlay so that the size of colonies on nutrient agar was comparable to the size of 14-hr colonies on bicarbonate agar, it was possible to demonstrate lysis of sensitive strains. These results with sensitive strains are compatible with what would be expected if penicillinase, or some penicillin inhibitor, was produced during growth. The mechanism of resistance of highly resistant strains (4-8, 10-24) appeared to be somewhat different; growth occurred in the presence of penicillin even when there was no visible colony development at time of overlay.

Studies on outgrowth from spore inocula in media containing penicillin contributed to an understanding of factors involved in penicillin resistance. In previous outgrowth studies, with small inocula (100 spores) of sensitive strains, 0.01-0.03 units penicillin/ml inhibited growth in tryptose thiamine broth, on nutrient agar and on bicarbonate agar. With large inocula (10^5-10^6 spores) significant differences in sensitivity of strains were observed in tryptose thiamine broth. In particular, considerably more penicillin was required to inhibit growth of the avirulent noncapsulated Weybridge strain than that of virulent strains. Other investigators have made similar observations. Bennett and his co-workers (Antibiotics and Chemotherapy 9:115, 1969) reported that avirulent laboratory strains were more than 1,000 times more resistant to penicillin than virulent strains. Therefore it was necessary to determine whether the presence of avirulent mutants in large inocula might be responsible for differences in the apparent resistance of virulent strains. By use of bicarbonate agar it would be possible to detect the presence of noncapsulated mutants.

Spore concentrations of 10^5-10^6 spores of virulent parent and mutant strains were plated on nutrient and bicarbonate agar containing 0.1, 1, 10 and 100 units penicillin/ml. Only highly resistant strains (4-8, 10-24) were able to grow on nutrient agar containing penicillin and at penicillin concentrations greater than 0.1 units/ml; growth consisted of encapsulated cells except at the 100 unit/ml penicillin concentration (strain 4-8). All strains grew on bicarbonate agar containing 0.1 unit penicillin/ml but, with the exception of strains 4-8 and 10-24, growth represented less than 0.1% of the inoculum and colonies of noncapsulated cells were usually present. The proportion of the plate population that was noncapsulated varied with the strain. When several plates of the same strain showed differences in amount of growth, increased growth was associated with an increase in the number of colonies of noncapsulated cells. Microscopic (430X) examination of colonies indicated that noncapsulated cells were not affected by penicillin but encapsulated cells, especially those at the edge of colonies, were twisted or showed some string-of-pearls reaction. The antibiotic appeared to favor selection of noncapsulated mutants in the original inocula; with inocula of 10^5-10^6 spores mutants could be detected if they represented only 0.001% of the population.

In Vitro Studies with Antiserum. A series of experiments were done to test whether differences in strains V1b-189 and NH-6 could be detected by culture in homologous and heterologous hyperimmune burro sera. The most noteworthy observation from these studies was the suppression of growth by sera from untreated burros but not by sera obtained from the same animals after immunization.

Neither rate of growth nor final cell yield of any strain was altered by the presence of 10% burro serum in medium that allowed optimum growth or in minimal defined medium, but the organisms always grow in long chains. Some cells of the Weybridge strain became spherical and appeared to degenerate in all sera; the same effect was noted with strain NH-6 but only with preimmune

sera, not with hyperimmune sera. Germination, growth and final cell yield in minimal, defined medium were markedly stimulated when any burro serum was in 20% concentration; the only serum effect on individual cells, as observed by microscopic examination, was the formation of a capsule by some cells. In 100% serum cultures germination rate was the same as in 20% serum cultures but growth occurred only with virulent strains and only in hyperimmune sera. Although the avirulent Weybridge strain germinated rapidly, all organisms were nonviable within 4 hr after inoculation. The lethal serum activity was resistant to heating for 30 minutes at 56 C.

This paradoxical effect of pre-immune and hyperimmune sera may be similar to some bacteriolytic effects reported by various investigators with other organisms (Bact Rev 21:273, 1957). In these studies addition of specific immune serum occasionally decreased the lytic activity of normal serum. The heat-resistant C'_4 fraction of complement seemed to be required in the system.

Growth in Defined Media. Development of a chemically defined medium was described in the previous FY 1963 Annual Progress Report. Until very recently a medium containing 0.005M glycine, 0.001M L-alanine, 0.01M DL-lysine HCl, 0.025M L-glutamic acid, 0.01M glucose, 0.05M $(NH_4)_2SO_4$, 0.001M K_2HPO_4 and 0.00004M $MgSO_4$ appeared to be adequate to support growth of all strains of B. anthracis tested: 10 wild strains, 12 previously described mutants and 1 avirulent strain.

In liquid medium germination was essentially complete in 4 hr. The avirulent Weybridge strain had the most rapid germination rate; NH-6 strains, parent and mutants, and 2 mutants of V1b-189 (5C and 202B) had somewhat slower rates; and strain V1b-189, mutants 4-8 and 205A, and wild strains 107, 108, 922, 1133 and 7287 germinated slowly. The interval between completion of germination, as measured by return to heat sensitivity, and initiation of log phase growth was 2-4 times longer than that required for the same strains in tryptose thiamine broth, but the slope of the growth curves during the log phase was the same as in tryptose thiamine broth. A cell yield of 13-14 generations was obtained within 48 hr. At this time all wild strains showed good sporulation; mutant strains were not as well sporulated but spore yields were as good or better than yields from complex media. Capsules were formed by virulent strains in the presence of 0.025M $NaHCO_3$, but protective antigen and toxin could not be detected. On medium containing 2% Bacto agar, colony counts were generally higher than on the usual complex nutrient media.

Growth requirements for the avirulent Weybridge strain differed from those for virulent strains V1b-189 and NH-6. For example, for growth of the Weybridge strain glucose and glycine were required and tris(hydroxymethyl)-aminomethane (Tris) or 0.01M bicarbonate were inhibitory. With the virulent strains Tris could partially substitute for glycine and 0.05M bicarbonate was not inhibitory. The typical virulent strain, V1b-189, had no glucose requirement and NH-6, the virulent strain that killed immunized

guinea pigs, could utilize glycerol or D-ribose as substitutes for glucose. Further definition of growth requirements for individual strains must await solution of a problem that was encountered recently.

During the past month it was found that none of the strains was able to grow satisfactorily in liquid medium prepared with a new lot of L-glutamic acid from the same supplier (Nutritional Biochemicals Corp.). Substitution of the new lot of L-glutamic acid in solid medium containing 2% Bacto agar and 0.025M $NaHCO_3$, however, resulted in the same or better recovery from small inocula (50-100 spores) of 10 of the 23 strains than on the control medium. Only 40-75% recovery was observed for the other 13 strains. Apparently Bacto agar contained some growth requirement and/or stimulant similar to the impurity in the old lot of L-glutamic acid. Thiamine, purines, pyrimidines, glutamine and glutathione did not stimulate growth but there is some evidence that the Cl^- may be implicated.

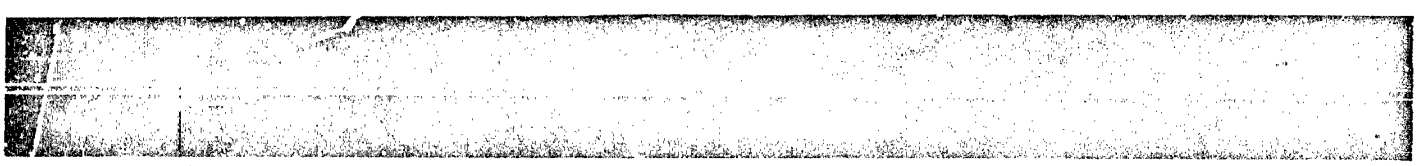
Summary and Conclusions:

Encapsulated mutant strains isolated from a monkey inadequately treated with penicillin and from immunized guinea pigs were virulent for guinea pigs and showed no reversion to parent type in vivo. As has been demonstrated with typical virulent strains, animals surviving challenge were not resistant to a subsequent challenge. None of the present cultural methods, however, provided an effective in vitro means of evaluating the relative virulence of encapsulated strains. Limited studies suggested that estimates of relative virulence derived from titrations in guinea pigs probably would not be the same as estimates from titrations in mice.

In vitro methods for detecting strain differences in penicillin sensitivity are complicated by production of penicillinase, or a penicillin inhibitor, by many strains of B. anthracis. When several methods of testing penicillin sensitivity were compared, only selected mutants that were isolated after inadequate in vivo treatment with penicillin were significantly more resistant than the parent strain in all tests. Results of studies with large inocula indicated that for many strains in vitro resistance may be related to the presence of noncapsulated mutants in the inoculum.

Serum culture could not be used as a means of distinguishing between virulent strains. In single passage experiments growth of virulent strains was not affected by homologous or heterologous hyperimmune burro sera but was completely suppressed by pre-immune sera from the same animals. Growth of an avirulent strain was inhibited by either pre-immune or hyperimmune burro sera. In no instance was germination affected adversely, but germinated cells were killed within 4 hr. Whole complement was not involved in the lethal effect.

Investigation of differences in requirements for growth still appears to be the most productive approach to the problem of differentiating between strains in vitro. Recent difficulties arising from the presence of



impurities in a preparation of crystalline L-glutamic acid have delayed the progress of these studies.

Publications:

None.

ANNUAL PROGRESS REPORT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-03:	Laboratory Identification of Biological Warfare Agents
Study No. -20-02:	In vitro-produced Anthrax Toxin
Reporting Installation:	U. S. Army Medical Unit Fort Detrick, Maryland
Division:	Bacteriology
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Authors:	Andrew J. Gaspar, Lt Commander, USN Martha K. Ward, Captain, USPHS Ralph G. Kanode, Jr. Hugh B. Tresselt, Ph.D.
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-03: Laboratory Identification of Biological Warfare Agents

Study No. -20-02: In vitro-produced Anthrax Toxin

Reporting Installation: U. S. Army Medical Unit
Fort Detrick, Maryland

Division: Bacteriology

Period Covered by Report: 1 July 1963 to 30 June 1964

Professional Authors: Andrew J. Gaspar, Lt Commander, USN
Martha K. Ward, Captain, USPHS
Ralph G. Kanode, Jr.
Hugh B. Tresselt, Ph.D.

Reports Control Symbol: RCS-MEDDH-288

Security Classification: UNCLASSIFIED

The biological and serological activity of crude anthrax toxin has been concentrated 500-1000 fold by a relatively simple ultrafiltration procedure. Yields of rat lethal activity seem to vary with different methods of harvest. The backwash method of harvest has usually yielded 50-100% of the total rat lethal activity present in the crude toxin used for ultrafiltration. Recovery of guinea pig skin edema activity has been more variable and rarely has reached 50% of the starting material. Recovery of antigenic activity as detected by agar gel diffusion has often exceeded a calculated 100%. Possible reasons for these observations are being investigated.

Storage studies indicate that original activity of the concentrated toxin is maintained for as long as 2 months in material that is shell frozen and stored at -20 C.

Preliminary work on the characterization of toxin using electrophoresis, immunoelectrophoresis, chemical fractionation and chromatography methods has been initiated. The production of a large pool of concentrated material for definitive chemical, biophysical and biological studies has begun.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-03: Laboratory Identification of Biological Warfare Agents

Study No. -20-02: In vitro-produced Anthrax Toxin

Description:

To study the exotoxin produced in vitro by Bacillus anthracis, to define the conditions required for its elaboration, to develop methods for concentration and purification without prior separation into its component parts, to study its chemistry and ultimately, to study its mode of action and role in the pathogenesis of the disease.

Progress:

Preliminary work described in the FY 1963 Annual Progress Report suggested that it might be feasible to concentrate and purify crude anthrax toxin by ultrafiltration or column chromatography or a combination of these methods without prior separation into its component parts. Work during this report period has therefore been concentrated on the development of procedures for obtaining concentrated whole toxin in as pure a form as possible.

Initial attempts employed lyophilization of crude culture filtrates sterilized by Millipore filtration and subsequent passage of reconstituted material through Sephadex G-100 columns. For successful lyophilization filtrates had to be dialyzed or otherwise desalted before freeze-drying. Although the results of this work were somewhat encouraging in that a single protein peak containing toxic activity could be obtained by passage of the dried material through Sephadex columns, there was considerable loss of total toxic activity during the processing; the Sephadex-treated material appeared to be less stable than the untreated material. In addition, the required dialysis of column desalting before lyophilization proved to be cumbersome. This method was finally discarded in favor of a simpler ultrafiltration procedure.

The ultrafiltration apparatus is shown in diagrammatic form in Figure 1. Using this method, volumes of 40-50 L of crude toxin could be concentrated 500-1000-fold conveniently in a matter of several days with limited personnel effort. Several methods for harvesting the ultrafiltrate residue containing the concentrated toxin (hereafter referred to as UFR) have been investigated: (1) stripping the UFR from the dialysis sacs after the filtration had proceeded to the point where only a very small volume of material remained in the sacs; (2) rinsing the sacs with Tris buffer after processing to near

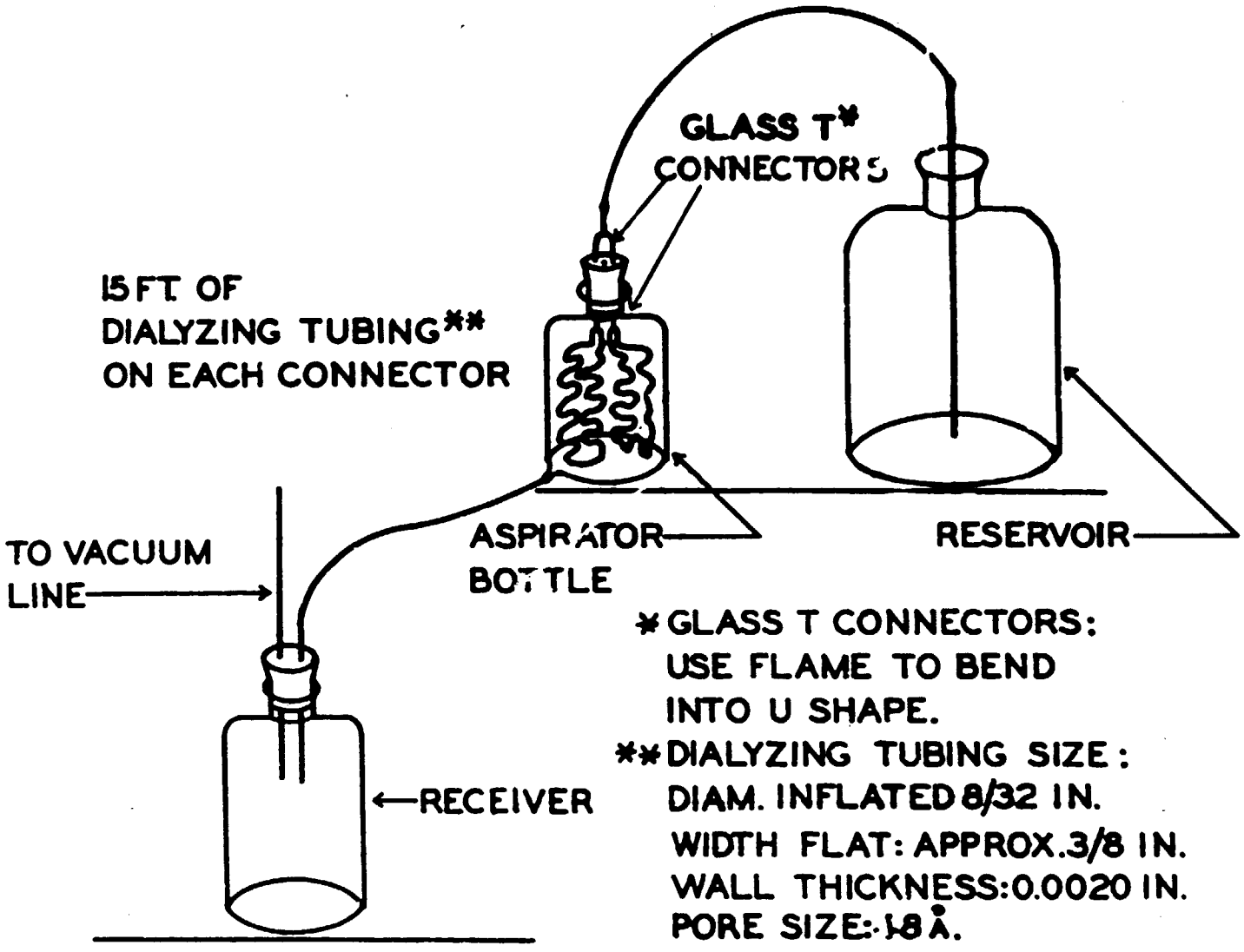


FIGURE I. ULTRA FILTRATION APPARATUS.

dryness, and (3) backwashing by placing the sacs after filtration into a large container of buffer and allowing back diffusion of buffer before stripping out the fluid from the sacs. In some experiments 2 methods were compared on aliquots of crude toxin and in other combinations of methods were used.

Sephadex treatment of the UFRs resulted in some loss of activity and apparently removed little extraneous nitrogenous material from these preparations. In addition, the activity of Sephadex-treated UFR toxin was again apparently less stable as had been noted previously with lyophilized batches of toxin. Consequently Sephadex treatment of UFR toxin has now been abandoned.

All preparations were assayed for lethal activity in rats and for edema-forming activity in the skin of the guinea pig. The in vitro antigenic activity was determined by the agar diffusion method. Whenever it was possible to do so, biological assays were performed on the day of harvest. Estimated percentage of recoveries of biological activity and titers of antigenic material in gel diffusion plates were calculated in terms of total activity of crude material on a volume-dilution basis. Tris buffer (0.005M pH 8.5) has been used throughout for harvesting and diluting concentrated toxin.

The calculated yields of activity obtained in 10 concentrated preparations of toxin from 8 lots of crude material are summarized in Table I. Also noted are the yields of activity obtained by backwashing dialysis sacs in Tris buffer after the harvest of UFR in 4 instances. Each step in the production of the crude toxin and in the harvest and assay of UFR was controlled as carefully as possible.

Recovery of Rat Lethal Activity. In general, the recovery of rat lethal activity was much better than guinea pig skin edema activity and was less variable from lot to lot harvested by the same method. The recovery of total activity in 4 of 5 preparations harvested by the backwash method ranged between 50 and 100%. The errors inherent in the volume-dilution and assay procedures make 2-fold differences of questionable significance. The one backwash preparation (30B) which yielded less than 50% of total lethal activity was held at 4 C for 6 days before assay. Although the lethal activity of crude toxin is generally quite stable for several weeks at 4 C, we now have information to indicate that the concentrated preparations are not as stable at this temperature as the original material. This point is under further investigation.

Only 1 of the 5 UFRs harvested by the stripping or rinse method gave recoveries of as much as 50% of the total activity in the starting material. Several factors may have affected these results: (1) the high recoveries of activity in the backwashes after harvest by these methods suggest the more obvious reason, i.e., the difficulty in removing all of very small volumes of fluid from 15-30 feet of dialysis tubing by this method, and (2) all of these preparations were more concentrated than those harvested by the backwash method even after addition to buffer to rinse the tubing. If, as appears

TABLE I. YIELDS OF ACTIVITY IN ANTHRAX TOXIN CONCENTRATED BY ULTRAFILTRATION AND HARVESTED BY SEVERAL DIFFERENT METHODS

BATCH NUMBER CRUDE TOXIN	METHOD OF UFR HARVEST AND VOLUME RECOVERED	FINAL CONCENTRATION OF UFR IN TERMS OF ORIGINAL	CALCULATED YIELDS OF ACTIVITY AS % OF TOTAL IN CRUDE TOXIN			NOTES
			Assay Method[a]	UFR[b]	Rinse or backwash after harvest	
35	Backwash 9.2 ml	489X	RL	100.0	<5.0	4 days filtration. Overnight backwash. First assay day of harvest. Second assay after 3 days at 4 C indicated more than 50% loss of activity.
			GPE	30.0	5.0	
			GD	320.0	27.0	
33	Backwash 70.0 ml	400X	RL	50.0	22.0	3 days filtration. Overnight backwash. Assay after 2 days at 4 C.
			GPE	2.5	0.1	
			GD	80.0	15.0	
32	Backwash 14.0 ml	657X	RL	50.0		5 days filtration. Overnight backwash. Assay day of harvest.
			GPE	5.0		
			GD	100.0		
30	(a) Stripping and rinse: 0.4 ml stripped. Buffer rinse to 4.7 ml	1300X	RL	< 25.0	1.0	5 days filtration. Held at 4 C for 6 days before assay.
			GPE	5.0	0.05	
			GD	40.0	9.0	
	(b) Backwash 6.1 ml	1000X	RL	20.0		
			GPE	2.5		
			GD	40.0		

TABLE I. YIELDS OF ACTIVITY IN ANTHRAX TOXIN CONCENTRATED BY ULTRAFILTRATION AND HARVESTED BY SEVERAL DIFFERENT METHODS (Continued)

BATCH NUMBER CRUDE TOXIN	METHOD OF UFR HARVEST AND VOLUME RECOVERED	FINAL CONCENTRATION OF UFR IN TERMS OF ORIGINAL	CALCULATED YIELDS OF ACTIVITY AS % OF TOTAL IN CRUDE TOXIN			NOTES
			Assay Method[a]	UFR[b]	Rinse or backwash after harvest	
28	Stripping 21.0 ml	846X	RL	25-50.0	18.0	3 days filtration. Assay day of harvest. Bacterial contamination.
			GPE	40.0	0.2	
			GD	80.0	23.0	
27	(a) Stripping: 0.4 ml. Buffer added to total of 8.7 ml.	1000X	RL	10.0	52.0	4 days filtration. Overnight backwash. Assay day of harvest.
			GPE	10.0	26.0	
			GD	20.0	211.0	
	(b) Backwash 12.8 ml	680X	RL	50.0		
			GPE	20.0		
			GD	320.0		
26	Stripping 2.5 ml. Buffer to 8.5 ml.	1000X	RL	25.0	< 0.3	4 days filtration. Assay day of harvest.
			GPE	40.0	10.0	
			GD	40.0	337.0	
25	Stripping 4.7 ml	900X	RL	> 50.0		5 days filtration. Assay day of harvest.
			GPE	27.0		
			GD	54.0		

a. RL equals rat lethal activity.
GPE equals guinea pig skin edema activity.
GD equals antigenic activity as determined by agar gel diffusion.

b. UFR equals ultrafiltrate residue, i.e., toxin concentrated by ultrafiltration.

to be the case, concentrated toxin is less stable than the more dilute crude material, this could have accounted partially for lower yields.

Recovery of Guinea Pig Skin Edema Activity. Yields of edema-producing activity in the UFR preparations rarely reached 40-50% and were more variable from lot to lot than yields of lethal activity regardless of method of harvest. The effect of method of harvest is not so apparent a possible explanation in this case as in the instance of rat lethal activity. It has been observed by other workers, as well as in this laboratory, that edema-producing activity is not as stable at 4 C (even in crude material) as is the rat lethal activity. The time required for dialysis may account in part for some of this loss. However, the results of assays on crude material held in the cold room for the same period of time as that required for the ultrafiltration indicate that the loss of activity on standing does not account for the relatively poor recoveries in UFR preparations or for the lot variations. In the search for possible explanations for these observations, the possibility that some of the material responsible for edema production passed through the dialysis sac into the filtrate was considered. The filtrates were tested for edema-producing activity and none was detected. Samples of 1-5 L of several batches of filtrate were passed through glass filters which were then eluted with bicarbonate buffer according to the method of Thorne, et al (J Bact 79:450, 1960). By this method "filter factor" preparations were obtained which are described briefly below. This portion of the study is still in a preliminary stage, and it has not yet been determined whether the activity which can be recovered from the filtrates accounts for a significant proportion of the loss during the processing. More work on this point is underway. The observation that any detectable amount of active material can be recovered from the ultrafiltrates, however, is of considerable interest since it suggests that the factors responsible for biological activity must be of variable molecular weight and/or configuration.

Results of titrations of backwashes collected from the dialysis tubing after the UFR had been harvested by the stripping procedure in some initial experiments suggested that the edema factor might be selectively adsorbed on the tubing during the processing, since more edema activity was observed in the backwash than in the UFR. However, this did not prove to be a consistent finding as can be seen in Table I.

The work of Stanley and Smith (J Gen Microbiol 26:49, 1961; Ibid 31: 329, 1963) with combinations of purified Factors I, II and III, suggests still another possible explanation for the relatively poor recoveries of edema activity in the UFR. These workers demonstrated: (1) that the presence of Factor III (lethal factor) inhibited edema production by combinations of Factors I and II, and (2) in later work, that the immunogenicity of combinations of Factors I and II was markedly inhibited by the presence of Factor III under certain conditions. It therefore seems possible that this inhibition phenomenon may be involved in our concentrated preparations.

In addition, ultracentrifuge studies included elsewhere in this report* suggest the presence of aggregates of material in the UFR, and that the proportion of aggregate to other components varies in different preparations. It is not possible at the present time with the data available to correlate this information with biological activity of specific batches. Further work to clarify this point is underway. It is also hoped that immunoelectrophoresis and chromatography studies initiated recently will eventually provide further clarification.

Recovery of Antigenic Activity as Measured by Gel Diffusion. The recovery of plate units of activity as measured by titers of various preparations in gel diffusion tests has, in general, been more consistent and complete than recovery of biological activity. With one exception (Batch No. 308) recovery has been complete, and, more often than not, the total calculated recovery in UFR plus backwash has exceeded a calculated 100%. It does not seem reasonable that technical error would always result in higher recoveries than expected. No satisfactory explanation for this observation is at present apparent. Most of the quantitative work done on factors affecting the appearance and titer of precipitin lines in the technique used has been done with relatively pure systems and with specific homologous antiserums. The materials used obviously contain a number of antigens and the only standard antiserum available was prepared by immunization with a living spore vaccine. It has been observed very frequently that only one line of precipitation can be detected in tests with crude toxin, and that the maximum number detected was 2. Work with purified components of toxin has demonstrated that the 3 known components which differ in their biological activity are serologically distinct. If that be the case then, at least one serological component has never been recognized in our crude toxin preparations, although the biological activity associated with the component was present. Although a great deal of work has been done during the course of this study in attempts to associate biological activity with specific serological components in our preparations no such association has been demonstrated. It is quite possible that aggregation, dissociation of aggregates, concentration effects, differing salt content of crude and concentrated preparations or a variety of other factors may affect the results of titrations and attempts to determine identity of lines of precipitate in our materials. A great deal more study will be needed to answer the questions which have arisen. As time and available materials permit, attempts to isolate and identify the 3 components of toxin as prepared separately by other workers will be made, using the UFR preparations as starting material.

Recovery of Dialyzable, Antigenically Specific Material from Ultrafiltrates. As mentioned above, in the search for the source of loss of edema activity during ultrafiltration, the filtrates of several batches were examined for biological and serological activity. In one or two instances leaks in connections of the dialysis tubing were recognized after an unusually rapid flow was observed. These filtrates were lethal for rats, and in one case some serological activity was observed. However, on other occasions where no leak could be detected and no biological or serological activity could be demonstrated in the filtrate, loss of edema activity was still obtained.

* Study 01-20-05.

Samples of filtrate from 2 such batches were filtered through glass filters and the filters then eluted with bicarbonate buffer as in preparation of filter factor by Thorne, et al (Op. Cit.). In both instances specific antigenic activity was recovered in the filter eluate. Results of preliminary studies on this material may be summarized as follows:

a. Three lines of precipitate were observed in gel diffusion plates using the standard antiserum.

b. None of these components identified with the line formed by known, partially purified protective antigen.

c. At least 2 of the 3 identified with lines formed by known filter factor prepared from crude toxin by the method of Thorne.

d. In certain combinations with known protective antigen (determined by box titration) rat lethal and skin edema activity were demonstrated.

Storage Stability of Concentrated Toxin. These storage studies are of necessity a part of a long term study, and most of the information available has been obtained with Sephadex-treated material. It has been demonstrated that the untreated UFR is generally more stable than the Sephadex-treated material in a few parallel observations. It is therefore reasonable to assume, for the present at least, that untreated UFR will be as stable under the same storage conditions as the Sephadex-treated material has proved to be.

Results of assays made on the Sephadex G-100 treated UFR preparations after periods of storage for at least 2 months indicate that:

a. The material is not stable at 4 C.

b. The toxin shows no significant loss of activity after 2 months storage under the following conditions:

(1) Quick shell freeze: storage at -20 C.

(2) Shell freeze and lyophilization, with storage at room temperature, 4, -20 or -70 C. There was a slight suggestion that storage at -20 C after lyophilization was slightly better than at room temperature, 4 or -70 C although the differences are of questionable significance.

It now appears that it will be feasible to make a large pool of toxin for definitive chemical biophysical and biological studies by accumulation of material from a number of batches prepared and stored quick frozen at -20 C. This project is underway.

Characterization of Toxin and Its Components. Preliminary studies, using electrophoresis, immunoelectrophoresis, chemical fractionation and

chromatography techniques have been initiated recently. It is hoped that this work will provide answers to many questions regarding the chemical nature of toxin and the apparently complex interrelationships of its components as prepared and described by others.

Summary and Conclusions:

An ultrafiltration method for preparation of concentrated anthrax toxin without prior separation into its 3 known components has been developed.

The results of studies on 10 lots of this concentrated toxin (UFR) indicate that the method of harvest is important for recovery of rat lethal activity. Yields of 50 to 100% of lethal activity calculated in terms of the total activity of the crude material used for concentration have been obtained in 4 or 5 UFR preparations harvested by the backwash method. Recovery of guinea pig edema activity has rarely exceeded 50% and proved to be more variable from lot to lot regardless of method of harvest. Recovery of antigenic activity has been more complete and frequently exceeded a calculated 100%. Further work to determine the reasons for the loss of guinea pig edema activity and the excessively high antigenic activity is in progress.

Dialyzable, specially active material which can be eluted with bicarbonate buffer from glass filters has been demonstrated in the ultrafiltrates from concentrated preparations. In appropriate combinations with known protective antigen toxic activity of this material could be demonstrated. So far as we are aware this is the first time that the presence of dialyzable, active fractions has been demonstrated.

Storage studies indicate that concentrated toxin retains its activity after 2 months of storage at -20 C if it is shell frozen prior to storage.

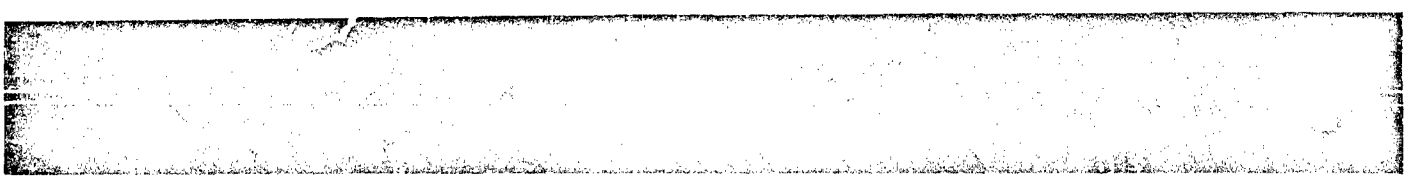
Work on the production of a large pool of concentrated toxin for definitive chemical, biophysical, immunological and biological studies has been initiated. Preliminary work on the characterization of the concentrated toxin has begun.

Publications:

None.

ANNUAL PROGRESS REPORT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-03:	Laboratory Identification of Biological Warfare Agents
Study No. -30-01:	Tissue Culture Studies with Rickettsiae.
Reporting Installation:	U. S. Army Medical Unit Fort Detrick, Maryland
Division:	Medical
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Author:	Erich D. Ryll, Major, MC
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED



ABSTRACT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-03: Laboratory Identification of Biological Warfare Agents

Study No. -30-01: Tissue Culture Studies with Rickettsiae.

Reporting Installation: U. S. Army Medical Unit
Fort Detrick, Maryland

Division: Medical

Period Covered by Report: 1 July 1963 to 30 June 1964

Professional Author: Erich D. Ryll, Major, MC

Reports Control Symbol: RCS-MEDDH-288

Security Classification: UNCLASSIFIED

Coxiella burnetii has been selected as a model for tissue culture studies of rickettsiae. Yolk sac stocks of Phases I and II of the Henzerling strain have been prepared and are now being tested. Examination of infected monolayers in bottles have shown cellular changes not seen in the controls. Cover slip methods are now being utilized because of their greater versatility.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-03: Laboratory Identification of Biological Warfare Agents

Study No. -30-01: Tissue Culture Studies with Rickettsiae.

Description:

Tissue culture methods will be used to attempt early clinical identification, to perform a neutralization test, and to investigate interference and phase variation of Coxiella burnetii.

Progress:

Twenty per cent yolk sac suspensions of Phases I and II of the Henzerling strain of Coxiella burnetii were obtained from Dr. Joseph Lowenthal, Biologics Division, Walter Reed Army Institute of Research. The pedigrees were:

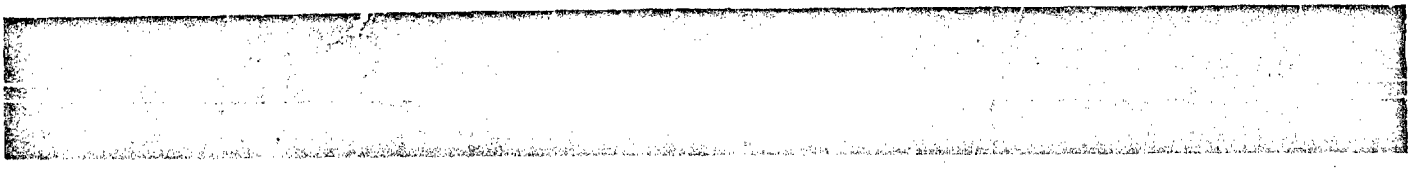
Henzerling Phase I: GP6/YS21/GP1/YS4
Henzerling Phase II: GP6/YS22

Stocks of both phases were prepared by yolk sac inoculation of 7-day embryonated eggs. Viable embryos were harvested 7-8 days postinoculation utilizing Snyder I solution as a diluent; yolk sacs were ground in a Waring blendor and placed in vaccine stoppered ampules.

These stocks were titered in 7-day embryonated eggs via the yolk sac route with the following results according to the Reed-Muench formulation:

Henzerling Phase I: (GP6/YS21/GP1/YS4/YS1) = $10^{6.5}LD_{50}$
Henzerling Phase II: (GP6/YS22/YS1) = $10^{3.8}LD_{50}$

L cells (mouse fibroblast) were utilized in the initial tissue culture experiments. Cells were grown in 8-oz prescription bottles with Eagles solution and 20% horse serum and maintained in medium 199. Falcon plastic bottles and 4-oz prescription bottles were inoculated with a 10^{-3} dilution of the stock Phase II. These containers were found unsuitable for high power microscopy. Examination of the infected cell sheets in Falcon bottles at a magnification of 450X, however, revealed certain changes when compared with controls. The infected monolayers became heavily granular, darker in appearance, and showed some vacuole formation. Some cells burst and eventual detachment of the monolayers was noted. Control bottles exhibited only a slight granularity with no significant detachment. In order to use higher magnification, cover slip techniques are being evaluated.



The rickettsial stocks are being titered in guinea pigs for ID_{50} and LD_{50} determinations.

Summary and Conclusion:

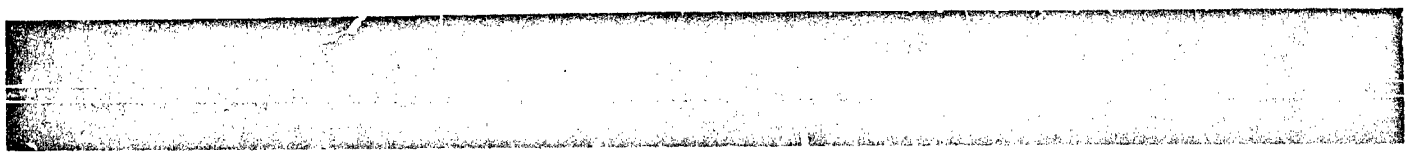
C. burnetii has been selected as a model for tissue culture studies of rickettsiae. Yolk sac stocks of Phases I and II of the Henzerling strain have been prepared and are now being tested. Examination of infected monolayers in bottles have shown cellular changes not seen in the controls. Cover slip methods are now being utilized because of their greater versatility.

Publications:

None.

ANNUAL PROGRESS REPORT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-03:	Laboratory Identification of Biological Warfare Agents
Study No. -40-01:	Development of Inactivated Serologic Reagents
Reporting Installation:	U. S. Army Medical Unit Fort Detrick, Maryland
Division:	Virology
Period Covered by Report:	1 July 1963 to 30 June 196[illegible]
Professional Authors:	Helen H. Ramsburg Sherman E. Hasty
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED



ABSTRACT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-03:	Laboratory Identification of Biological Warfare Agents
Study No. -40-01:	Development of Inactivated Serologic Reagents
Reporting Installation:	U. S. Army Medical Unit Fort Detrick, Maryland
Division:	Virology
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Authors:	Helen H. Ramsburg Sherman E. Hasty
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

Noninfectious serologic test antigens were prepared with Langat (TP-21) and Mayaro viruses. Certain factors regarding antigen preparation and its use in the hemagglutination-inhibition test are described.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-03: Laboratory Identification of Biological Warfare Agents

Study No. -40-01: Development of Inactivated Serologic Reagents

Description:

To provide noninfectious antigens for use in the hemagglutination-inhibition and complement-fixation tests in the serological diagnosis of arbovirus infections.

Progress:

The use of beta-propiolactone (BPL) for preparation of inactivated serologic test antigens has continued. Preliminary studies with Mayaro, Langat (TP-21), and yellow fever viruses described last year have been extended.

Initial attempts to prepare antigens with these agents were unsuccessful. Subsequent titration of the mouse brain suspensions showed the infectious virus titers to be low. Accordingly, serial passage with concurrent titrations of each virus was carried out. Suckling mice, 4-5 days of age inoculated by the intracerebral (IC) route were employed.

With TP-21 virus there was a gradual increase in titer obtained with each passage. This increase approximated 1 log per passage so that after 5 successive passages in 4-5 day old suckling mice a titer of $10^{-9.5}$ suckling mouse median intracerebral lethal doses ($SMICLD_{50}$) of virus per 0.02 ml was attained.

Serial passage of Mayaro virus did not result in an increase in titer of the same degree as with TP-21. Virus titers varied with the same passage level as well as with successive passages. It was found that by employing relatively large quantities of virus for inoculum, titers of approximately $10^{-8.5}$ could be obtained. This level was found to be adequate for antigen production.

In contrast to the other viruses, only limited serial passages with yellow fever virus were required to achieve high titers.

Two methods of extraction were employed in preparation of the antigens for Mayaro and TP-21.

Extraction with acetone and ether was unsatisfactory. Antigens prepared in this manner had low hemagglutinin titers and were unstable when used in the hemagglutination-inhibition (HAI) test. In contrast, extraction with sucrose-acetone yielded antigens which were both stable and reactive in adequate titer.

Coincident with the extraction experiments, optimal pH values for the final antigens were established. The values for Mayaro and TP-21 were pH 7.1-7.4 and 7.8-7.9 respectively.

Previous studies with other arbovirus antigens had indicated that both pH and temperature of incubation influenced hemagglutination. The earlier results had been obtained with both live and BPL-treated antigens. Insofar as Mayaro virus was concerned, the values described (Annual Report, USAMU, FY 1963) for other Group A arboviruses were applicable.

Similar studies were performed with TP-21 antigen prepared by the 2 extraction procedures. The results showing the influence of test pH and incubation temperature are shown in Table I.

TABLE I. HEMAGGLUTININ TITERS OF 2 DIFFERENT TP-21 ANTIGENS UNDER DIFFERENT CONDITIONS OF pH AND TEMPERATURE OF INCUBATION

	TYPE OF ANTIGEN							
	Acetone-Ether				Sucrose-Acetone			
	pH of Antigen				pH of Antigen			
	7.1		7.8		7.3		7.9	
pH OF TEST	Temperature of Incubation				Temperature of Incubation			
	Room[a]	37 C[b]	Room	37 C	Room	37 C	Room	37 C
5.75	160[c]	(320)[d]	320	(640)	(160)	(640)	640	(1280)
6.35	320	<20	1280	<20	320	<20	2560	(80)
6.75	(640)	(20)	(1280)	(40)	(640)	40	(1280)	320

a. Incubation time of 2 hr at room temperature.
b. " " " 1 hr at 37 C.
c. Reciprocal of titer.
d. ( ) = Incomplete agglutination and/or slippage.

Maximum hemagglutinin was demonstrated with the two antigens of pH 7.8-7.9 when tested at pH 6.35 and incubated for 2 hr at room temperature.

These studies are to be extended to include a broader range of test conditions including pH, temperature and length of incubation, and stability of antigen under conditions of storage.

Summary and Conclusions:

Methods which have been developed for the preparation of noninfectious serologic test antigens for the viruses of Eastern, Western and Venezuelan equine encephalitis have been applied to the preparation of noninfectious serologic test antigens for the TP-21 and Mayaro viruses. The elimination of hazard to infection associated with the use of noninactivated reagents has been extended to two more viruses.

Publication:

1. French, George R., and McKinney, Robert W.: "Use of Beta-propiolactone in Preparation of Inactivated Arbovirus Serologic Test Antigens," J Immunol. In press.

ANNUAL PROGRESS REPORT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-03:	Laboratory Identification of Biological Warfare Agents
Study No. -40-02:	Development of Serologic Tests for Early Identification of Viral Diseases
Reporting Installation:	U. S. Army Medical Unit Fort Detrick, Maryland
Division:	Virology
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Authors:	Section I Helen H. Ramsburg Warren R. Brunton, Captain, VC Section II Robert W. McKinney, Major, MSC Helen H. Ramsburg Warren R. Brunton, Captain, VC
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-03:	Laboratory Identification of Biological Warfare Agents
Study No. -40-02:	Development of Serologic Tests for Early Identification of Viral Diseases
	Section I. Production of serologic test antigens.
Reporting Installation:	U. S. Army Medical Unit Fort Detrick, Maryland
Division:	Virology
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Authors:	Helen H. Ramsburg Warren R. Brunton, Captain, VC
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

Results are presented which indicate that the "normal" monkeys available for experimental studies have prior antigenic experience with a Group A arbovirus. This prior experience precludes the use of such animals in studies of antigenic analysis, cross protection, etc.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-03: Laboratory Identification of Biological Warfare Agents

Study No. -40-02: Development of Serologic Tests for Early Identification of Viral Diseases

Section I. Production of serologic test antigens.

Description:

To determine heterologous reactivity of antibody to selected members of the Group A arbovirus complex.

Progress:

Investigations in this laboratory have been concerned with a limited number of the Group A arbo viruses. However, only recently has there been an opportunity to expand into an examination of relationships between these agents.

Mayaro virus was employed in the initial experiments programmed. Because only limited information relative to its host range was available, the production of immune serum was combined with an examination of the susceptibility and response of the monkey to infection with the virus. It was anticipated that these animals would be employed in challenge studies with other Group A viruses.

Mayaro virus as infected suckling mouse brain was serially diluted over the range of 10^{-1} through 10^{-10}. One monkey per dilution was inoculated subcutaneously with 1.0 ml. Serum was obtained from all animals prior to inoculation and on days 1 through 11, 14, 23 and 28 following inoculation.

In initial serologic tests of day 1-9 sera with Mayaro antigen, results were obtained which indicated the existence of antibody prior to inoculation of virus. On the strength of these results, all pre-inoculation sera were tested with Mayaro, Western equine encephalitis (WEE), Eastern equine encephalitis (EEE), and Venezuelan equine encephalomyelitis (VEE) antigens. In these tests, the sera reacted only with WEE antigen.

In subsequent tests, all sera were tested with the 4 antigens. Serum hemagglutination-inhibiting (HAI) test results are presented in Table I.

TABLE I. HEMAGGLUTINATION-INHIBITING (HAI) ANTIBODY TITERS TO SELECTED GROUP A ARBOVIRUSES FOLLOWING INOCULATION WITH LIVE MAYARO VIRUS

MONKEY NO.	DILUTION OF MAYARO	DAY OF BLEEDING	RECIPROCAL HAI TITERS			
			VEE[a]	EEE[a]	WEE[a]	Mayaro
C-57	10^{-1}	Pre	<10	<10	160	<10
		7	<10	40	160	20
		10	20	40	160	160
		14	320	320	1280	2560
		23	160	160	640	2560
		28	80	80	320	1280
DD-8	10^{-2}	Pre	<10	<10	160	<10
		7	<10	<10	160	<10
		10	<10	10	80	40
		14	160	160	640	1280
		23	160	80	640	5120
		28	20	40	320	640
56	10^{-3}	Pre	<10	<10	80	<10
		7	<10	20	80	<10
		10	<10	20	80	40
		14	320	80	320	640
		23	320	160	320	1280
		28	80	40	160	640
N-25	10^{-4}	Pre	<10	<10	160	<10
		7	<10	20	320	<10
		10	<10	20	160	40
		14	40	80	640	640
		23	40	80	320	640
		28	20	80	320	640
CC-6	10^{-5}	Pre	<10	<10	320	<10
		7	<10	40	320	10
		10	<10	40	320	20
		14	20	80	640	640
		23	80	80	640	640
		28	40	40	640	640

TABLE I. CONTINUED

MONKEY NO.	DILUTION OF MAYARO	DAY OF BLEEDING	RECIPROCAL HAI TITERS			
			VEE[a]	EEE[a]	WEE[a]	Mayaro
C-30	10^{-6}	Pre	<10	<10	320	<10
		7	<10	40	320	<10
		10	<10	40	320	<10
		14	10	80	320	320
		23	40	160	640	640
		28	40	80	320	1280
B-37	10^{-7}	Pre	<10	<10	160	<10
		7	<10	20	160	10
		10	<10	20	320	40
		14	80	320	640	1280
		23	160	320	640	2560
		28	160	320	640	5120
C-59	10^{-8}	Pre	<10	<10	160	<10
		7	<10	20	320	20
		10	<10	10	320	40
		14	40	80	640	640
		23	160	160	320	2560
		28	80	80	320	2560
C-88	10^{-9}	Pre	<10	<10	640	<10
		7	<10	20	320	<10
		10	<10	10	320	<10
		14	<10	<10	640	<10
		23	<10	<10	320	<10
		28			160	
C-64	10^{-10}	Pre	<10	<10	320	<10
		7	<10	20	320	<10
		10	<10	10	160	<10
		14	<10	<10	320	<10
		23	<10	<10	160	<10
		28			80	

a. VEE - Venezuelan equine encephalomyelitis; EEE - Eastern equine encephalitis; WEE - Western equine encephalitis.

As may be seen, all animals had relatively high HAI antibody titers to WEE antigen prior to inoculation of Mayaro virus. Subsequent to inoculation, the pattern of antibody broadened with no significant change occurring in the WEE titers. This broadening of antibody to other Group A arboviruses is similar to that described by Casals (Am J Trop Med Hyg 12:587, 1963).

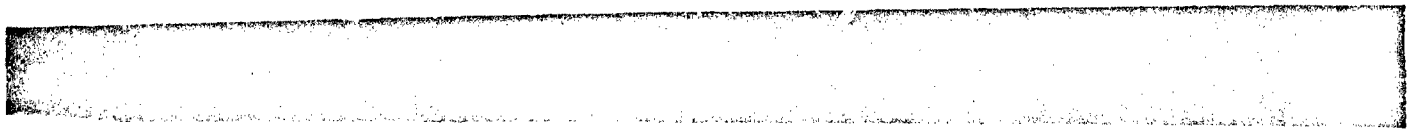
Although heterologous antibody was present at the time of inoculation it apparently did not influence the susceptibility of the monkey to infection with Mayaro virus. The results indicate that the monkey inoculated with the 10^{-8} dilution was infected, which compares with the titer of $10^{-8.5}$ obtained in 4-5 day old suckling mice inoculated intracerebrally with 0.02 ml of the same virus suspension.

Summary and Conclusions:

From these results, it is apparent that the animals had experienced infection with an agent antigenically related to those employed in the serologic tests. Therefore, the use of such animals in antigenic analysis, cross-protection studies, etc. is precluded.

Publications:

None.



ABSTRACT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-03:	Laboratory Identification of Biological Warfare Agents
Study No. -40-02:	Development of Serologic Tests for Early Identification of Viral Diseases
	Section II. Identification of viral strains.
Reporting Installation:	U. S. Army Medical Unit Fort Detrick, Maryland
Division:	Virology
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Authors:	Robert W. McKinney, Major, MSC Helen H. Ramsburg Warren R. Brunton, Captain, VC
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

Two strains of virus recovered from fatal cases of equine encephalitis in Peru, S.A., have been identified as Venezuelan equine encephalomyelitis (VEE) virus. No antigenic differences were demonstrable between these viruses and the prototype Trinidad strain of VEE.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-03: Laboratory Identification of Biological Warfare Agents

Study No. -40-02: Development of Serologic Tests for Early Identification of Vir ' Diseases

Section II. Identification of viral strains.

Description:

Identification of two strains of virus isolated from horses in Peru.

Progress:

In September, 1963, 2 strains of equine encephalitis virus were obtained from Dr. Jorge Olivares Cabera, Director, del Centro Nacional de Investigaciones de Patologia Animal, Lima, Peru.

These strains had been recovered from 2 horses dying with encephalitis. These animals were located in the Departments of Ica and Piura.

Both virus strains received as infected guinea pig brain in glycerol were labeled as follows:

Encefalomielitis Equina Virus Piura 16/9/63	Encefalomielitis Equina Virus Hofa Redonda Ica 16/9/63

The exact passage histories for the 2 strains identified as Ica and Piura are not available.

Each of the specimens was treated in the same manner. The tissue was ground with alundum in a sterile chilled mortar and suspended to 10% in phosphate buffered saline, pH 7.4, containing 1% inactivated normal rabbit serum (RSPBS). The suspension was clarified by centrifugation. Aliquots of the supernate were stored at -70 C for use as seed virus.

Each of 2 guinea pigs was inoculated intraperitoneally with 0.5 ml of the 10% suspension. Death occurred on days 6 and 7 after inoculation. At time of death the brains were re- ved, ground to 20% in RSPBS and stored at -70 C.

Suckling mice 4-5 days of age were inoculated intracerebrally (IC) with 0.02 ml of the 10% brain suspension. First deaths occurred on day 3 post-inoculation at which time the remaining animals were sacrificed and the brains pooled for antigen preparation. A crude antigen was prepared by grinding the brains in physiologic saline, pH 7.2. A ratio of 1 part brain to 2 parts saline was used. Protamine was added and the suspension clarified by centrifugation.

These antigens were employed in hemagglutination-inhibition (HAI) tests with immune serum for Eastern equine encephalitis (EEE), Western equine encephalitis (WEE) and Venezuelan equine encephalomyelitis (VEE) viruses. The results showed both strains to be VEE virus.

The same antigens were then tested in a block titration against VEE immune serum and compared to the Trinidad strain of VEE virus. The results are presented in Table I.

These tests do not reveal any antigenic differences between the Ica, Piura and prototype Trinidad strains. Reciprocal titrations were not performed.

Serum neutralization tests were performed with the 2 strains using serum obtained from animals following infection with either EEE, VEE or attenuated VEE virus. Both the Ica and Piura strains which titered 5.12 and 4.36 logs respectively were completely neutralized by each of the VEE sera. There was no evidence of neutralization of either strain by the EEE serum.

The neutralization test results gave evidence that the attenuated strain of VEE would provide protection against the 2 viruses. This was confirmed with protection tests performed with mice immunized with attenuated virus and challenged with either of the Peruvian strains.

NOTE: Although equine encephalitis was identified in Peru in 1930, the results reported represent the first definitive identification of viruses recovered from equines in Peru. A formalin inactivated chick embryo vaccine prepared with the 2 strains has been employed since about 1938 and except for an outbreak in 1952 in cows no significant amount of equine encephalitis has occurred. (Revisto del Instituto Nacional de Biologica Animal, vol 4, Dec 1953, Lima, Peru.)

Summary and Conclusions:

Two strains of virus recovered from horses dying of encephalitis in Peru have been identified as VEE. Results of serologic tests and protection studies show the 2 strains to be antigenically similar to the prototype Trinidad strain VEE virus.

Publications:

None.

TABLE I. COMPARISON OF 3 STRAINS OF VENEZUELAN EQUINE ENCEPHALOMYELITIS VIRUS BY THE HEMAGGLUTINATION-INHIBITION TECHNIQUE

IMMUNE SERUM TO TRINIDAD STRAIN - UNITS OF ANTIBODY	UNITS OF ANTIGEN 32	16	8	4	2	VIRUS STRAIN
4	0	0	0	0	0	
2	0	0	0	0	0	PIURA
1	0	0	0	0	0	
0.5	±	0	0	0	0	
0.25	+	+	±	0	0	
4	0	0	0	0	0	
2	0	0	0	0	0	ICA
1	0	0	0	0	0	
0.5	+	0	0	0	0	
0.25	+	+	±	0	0	
4	0	0	0	0	0	
2	0	0	0	0	0	TRINIDAD
1	0	0	0	0	0	
0.5	+	±	0	0	0	
0.25	+	+	±	0	0	

0 - Denotes inhibition of hemagglutination.

ANNUAL PROGRESS REPORT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-03:	Laboratory Identification of Biological Warfare Agents
Study No. -40-03:	Determination of Distribution of Viral Agents in Host Tissues Employing Radioactive Labeling Techniques
Reporting Installation:	U.S. Army Medical Unit Fort Detrick, Maryland
Division:	Physical Sciences Division
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Author:	Irving Gray, Colonel, MSC
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-03: Laboratory Identification of Biological Warfare Agents

Study No. -40-03: Determination of Distribution of Viral Agents in Host Tissues Employing Radioactive Labeling Techniques

Reporting Installation: U.S. Army Medical Unit
Fort Detrick, Maryland

Division: Physical Sciences Division

Period Covered by Report: 1 July 1963 to 30 June 1964

Professional Author: Irving Gray, Colonel, MSC

Reports Control Symbol: RCS-MEDDH-288

Security Classification: UNCLASSIFIED

Because of the transfer of the investigators to a higher priority study, no work was carried out during this fiscal year. This study has been terminated.

BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-03: Laboratory Identification of Biological Warfare Agents

Study No. -40-03: Determination of Distribution of Viral Agents in Host Tissues Employing Radioactive Labeling Techniques

Description:

To study the distribution in host tissues of viral agents employing radioactive labeling techniques.

Progress:

Because of the transfer of the investigators to a higher priority study, no work was carried out during this fiscal year. This study has been terminated.

Publications:

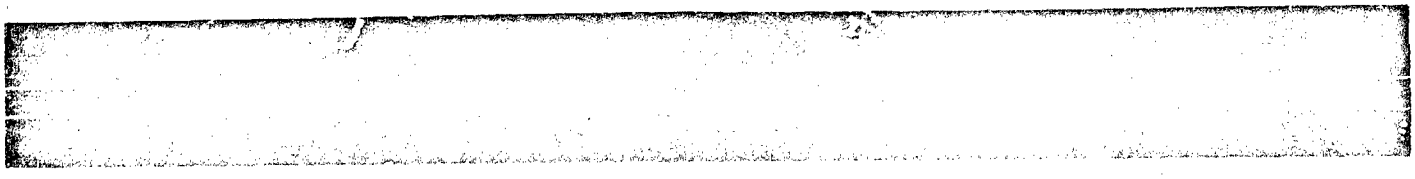
None.

Presentations:

None.

ANNUAL PROGRESS REPORT

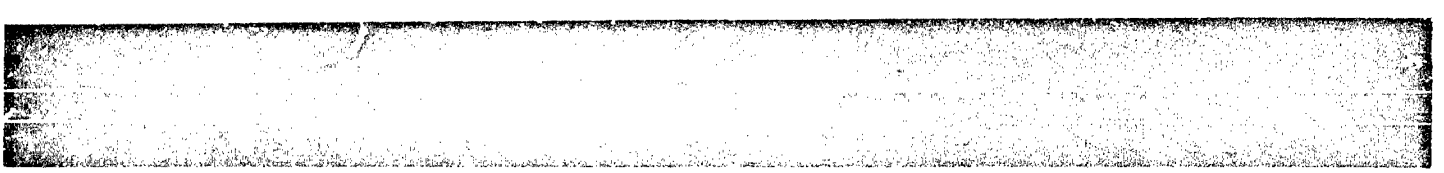
Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C622401A096-03:	Laboratory Identification of Biological Warfare Agents
Study No. -40-04:	Separation, Purification and Concentration of Arbovirus Agents and Antigen-Antibody Complexes
Reporting Installation:	U. S. Army Medical Unit Fort Detrick, Maryland
Division:	Virology
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Author:	Robert W. McKinney, Major, MSC
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED



ABSTRACT

Project No. 1C622401A096:	Medical Defense Aspects of Biological Warfare (U)
Task No. 1C62240 1A096-03:	Laboratory Identification of Biological Warfare Agents
Study No. -40-04:	Separation, Purification and Concentration of Arbovirus Agents and Antigen-Antibody Complexes
Reporting Installation:	U. S. Army Medical Unit Fort Detrick, Maryland
Division:	Virology
Period Covered by Report:	1 July 1963 to 30 June 1964
Professional Author:	Robert W. McKinney, Major, MSC
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

Investigations under this study were suspended because of lack of adequately trained personnel. It is anticipated that current recruitment will permit resumption during the next year.



BODY OF REPORT

Project No. 1C622401A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1C622401A096-03: Laboratory Identification of Biological Warfare Agents

Study No. -40-04: Separation, Purification and Concentration of Arbovirus Agents and Antigen-Antibody Complexes

Description:

To study arbovirus agents in purified preparations in terms of morphology or physical characteristics; to separate serologic and/or immunologic antigens from infectious virus particles; and to separate group and specific antigens in order to investigate antigen-antibody complexes in concentrated or purified forms.

Progress:

Investigations under this study were suspended because of lack of adequately trained personnel. It is anticipated that current recruitment will permit resumption during the next year.

Publications:

None.

APPENDIX A

U. S. ARMY MEDICAL UNIT GUEST LECTURE SERIES

DATE	GUEST LECTURER	TITLE OF PRESENTATION
20 Nov 63	Dr. Marion B. Sulzberger Technical Director of Research Office of The Surgeon General Washington, D. C.	The Opportunities and Obligations of Military Medical Research.
12 Dec 63	Dr. James G. Hirsch The Rockefeller Institute New York, New York	Motion Picture Studies on Phagocytosis and Degranulation in Polymorphonuclear Leukocytes.
16 Jan 64	Dr. David McK. Rioch Director, Division of Neuropsychiatry Walter Reed Army Institute of Research Washington, D. C.	Changing Concepts of Homeostasis.
13 Feb 64	Dr. Robert R. Wagner Department of Microbiology The Johns Hopkins University School of Medicine Baltimore, Maryland	Interferon: A Regulatory System in Viral Infections.
12 Mar 64	Dr. Vernon Knight Institute of Allergy and Infectious Diseases National Institutes of Health Bethesda, Maryland	Studies of Coxsackie A-21 Aerosol in Man.
2 Apr 64	Dr. Manfred M. Mayer [illegible]rtment of Microbiology The Johns Hopkins University School of Medicine Baltimore, Maryland	The Properties of Complement and Its Mode of Action.
26 Jun 64	Dr. Robert L. Squibb Professor of Nutrition in the Laboratories of Disease and Environmental Stress Rutgers, The State University New Brunswick, New Jersey	Some Nutritional and Biochemical Aspects of a Virus Infection.

APPENDIX B

U. S. ARMY MEDICAL UNIT PROFESSIONAL STAFF MEETINGS

DATE	LECTURER	TITLE OF PRESENTATION
18 Oct 63	Robert W. McKinney, Major, MSC Chief, Virology Division U. S. Army Medical Unit	A Summary of Observations and Experiences during Visit to Arbovirus Laboratories in South America and during Seventh International Congress of Tropical Medicine and Malaria in Rio de Janeiro.
22 Nov 63	Trygve O. Berge, Colonel, MSC Virology Branch, Armed Forces Institute of Pathology Walter Reed Army Medical Center Washington, D. C.	Etiology of Infectious Hepatitis.
17 Jan 64	Irving Gray, Colonel, MSC Chief, Physical Sciences Division U. S. Army Medical Unit	Biochemical and Physiological Responses Associated with the Administration of Staphylococcal Enterotoxin.
27 Feb 64	Martha K. Ward, Captain, USPHS Chief, Bacteriology Division U. S. Army Medical Unit	Studies on Anthrax Toxin.
	Dr. Virginia G. McGann Bacteriology Division U. S. Army Medical Unit	Preliminary Results of Investigations on the Antibody Response to Vaccination with Staphylococcal Enterotoxin. and Characterization of Strains of *Bacillus anthracis*.
24 Apr 64	Arthur L. Hogge, Jr., Colonel, VC Chief, Animal Assessment Division U. S. Army Medical Unit	Characteristics of Venezuelan Equine Encephalomyelitis in Swine.
	Ralph Z. Thomas, Major, VC Animal Assessment Division U. S. Army Medical Unit	Attenuated Venezuelan Equine Encephalomyelitis Virus in Pregnant Swine.

DATE	LECTURER	TITLE OF PRESENTATION
24 Apr 64 (Cont.)	William C. Day, Captain, VC Animal Assessment Division U. S. Army Medical Unit	Postexposure Prophylaxis and Treatment of Airborne Simian Tularemia with Novobiocin.
	Herbert L. Morton, Capt, VC Animal Assessment Division U. S. Army Medical Unit	Attenuated Venezuelan Equine Encephalomyelitis in Pregnant Swine.
	Melvin H. Davis, 1st Lt, VC Animal Assessment Division U. S. Army Medical Unit	Venezuelan Equine Encephalomyelitis in Lactating Sows.
	Ralph W. Kuehne Animal Assessment Division U. S. Army Medical Unit	The Efficacy of Viable Pasteurella tularensis Vaccine (LVS) Against Respiratory Challenge with Strains SCHU-S4 and SCHU-S5 in Macaca mulatta. and Evaluation of Living Vaccine Strain and TC-80 Vaccines Administered by the Respiratory Route.
28 May 64	John D. Marshall, Jr., Major, MSC Chief, Microbiology Division U. S. Army Medical Unit	Drug Resistant Malaria.
	Allan R. Larrabee, Captain, MSC Microbiology Division U. S. Army Medical Unit	Antigens from Pasteurella pestis.

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